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INTRODUCTION

Campylobacter species are generally microaerophilic bacteria but some species grow aerobically and some may grow under anaerobic conditions with an electron acceptor other than oxygen. They are either commensals or pathogens for humans and/or animals. The most common disease that is caused by these species is enteritis; other diseases include bacteremia, periodontitis, gingivitis, and Guillian Barré syndrome. Most *Campylobacter* infections are self-limiting and only incidental treatment with antimicrobial agents is indicated. Animal-to-human or human-to-human transmission is by the fecal-oral route or fecal-contaminated water.

Some bacteria enter an altered physiological state termed "viable but nonculturable" (VBNC). This is commonly defined as the inability to form colonies on a given solid medium yet giving indications of viability by other tests such as fluorescence staining with acridine orange. In some bacteria, stresses such as starvation, exposure to toxic chemicals, suboptimal temperature, or salinity within aquatic environments can induce sublethal physiological and structural changes which can greatly compromise their detection and enumeration on culture media, especially selective media. Most *Campylobacter* species not only enter the VBNC state but also change their morphological appearance from spiral to a coccoid form. Many researchers have attempted to resuscitate campylobacters from this VBNC coccoid state in vivo by feeding preparations of the coccoid form to experimental animals. Such experiments have seemingly resulted in resuscitation but they are controversial because the sample fed to the animal is not the same sample that was tested for the absence of culturable forms. If there were one or more spiral cells in a preparation of coccoid forms, these cells might multiply and lead to the false conclusion that resuscitation has occurred.

Many questions have been raised whether the VBNC forms of bacteria are degenerate forms or merely dormant resting forms. "Vital" staining and testing for oxygen uptake, RNA content, and ATP levels have been proposed for demonstrating that the coccoid forms are in a viable, dormant state. In some studies with campylobacters, chloramphenicol has been used to try to inhibit the conversion from spiral to coccoid form by preventing the synthesis of enzymes that might cause the spiral change to coccoid form, but such inhibition has not occurred. This has suggested that the specific enzyme(s) for

conversion may already exist in the spiral form before adding chloramphenicol. Therefore, studies of the conversion to the coccoid form may involve preexisting autolysins. The rigid peptidoglycan, located in the periplasm of a gram-negative bacterium, maintains the shape of the cell. During growth of the cell wall, autolysins (also called murein hydrolases) cut the old peptidoglycan in order that new peptidoglycan can be added. If these autolysins were to increase in activity beyond the level needed for the synthesis of peptidoglycan, they might cause conversion of the spiral form to the coccoid form.

Campylobacter upsaliensis is an organism that can cause various diseases in humans such as enteritis, breast abscess, bacteremia, or abortion. The source of infection is from dogs and cats. Although studies have been made of the viability of the VBNC of *Campylobacter jejuni* and *Helicobacter pylori*, and although there is a report that *C. upsaliensis* can convert to the coccoid form when exposed to air, there is no information about the optimal time and temperature for this conversion or even whether the coccoid form is culturable or nonculturable. In the present report, I have tested the viability of the coccoid form of *C. upsaliensis* in regard to growth on solid media, acridine orange staining, and oxygen uptake. I have also examined the integrity of ribosomal RNA (rRNA) in the coccoid form. Finally, I have attempted to demonstrate the presence of autolysins in both spiral and coccoid forms and to purify and characterize one autolysin in particular.

LITERATURE REVIEW

The genus *Campylobacter*

Campylobacter species have been described by Smibert (1978, 1984) and Penner (1988) and redefined by Vandamme et al (1991a, 1991b) as follows: slender, nonsporeforming, helically curved or straight gram negative rods that are 0.2-0.5 µm wide and 0.5-8.0 µm in length. Cells may appear as S-shaped or gull-winged when cells form short chains. Cells in old cultures may form coccoid/ spherical bodies. A polar membrane occurs at each end of the cells. This structure, which is composed of ATPase but whose functioni is unknown, also occurs in several other kinds of helical or vibrioid bacteria and is located and linked directly under the cytoplasmic membrane by bar-shaped linkers. Campylobacters are motile by means of either a unipolar flagellum or bipolar flagella that are 2 to 3 times the length of the cells. Darting movement is associated with gull-winged morphology, whereas a corkscrew-like movement in which rotation occurs along the long axis, is associated with spiral-shaped. Campylobacters are generally microaerophilic. They have a strictly respiratory-type of metabolism and can use O_2 as a terminal electron acceptors; however, they cannot grow at the level of O_2 present in air (21%). They are also capnophilic requiring at least 1-5% CO_2 for growth. Some species can grow anaerobically with either fumarate, formate + fumarate or H_2 + fumarate in the medium: the fumarate serves as the terminal electron acceptor and the formate or H_2 as the electron donor. Some species can use nitrate or trimethylamine oxide as a terminal electron acceptor for anaerobic respiration. Althogh campylobacters are chemoorganotrophs, they neither oxidize nor ferment carbohydrates. Instead they obtain energy from amino acids, the salts of tricarboxylic acid cycle (TCA) intermediates, the salts of other organic acids, or, in some species, H_2 or formate. All campylobacters are oxidase-positive (except *C. gracilis*), indole-negative, and nonpigmented. Catalase activity and nitrate reduction varies depending on the species. The G+C content of the DNA for members of the genus range from 29 to 46 mol % (Etoh et al. 1993, Fox et al. 1989, Harvey and Green wood 1983, Owen 1983, Owen and Leaper 1981, Ursing et al. 1983). There are fifteen species currently included in this genus. Some species are part of the normal flora of animals. Various campylobacters have been recognized for many years as infectious agents of

reproductive organs of sheep and cattle, and some species can cause disease in humans. This study will focus on *C. upsaliensis*.

Campylobacter upsaliensis

This organism was first isolated from the feces of diarrheic and asymptomatic dogs. It was initially considered a member of the "CNW group": catalase negative or weakly reacting *Campylobacter* (Olson and Sandstedt 1987, Sandstedt et al. 1983). Analysis of electrophoretic protein patterns and DNA relatedness studies showed that many CNW strains from humans belonged to the same group as the canine strains (Costas et al. 1987, Fox et al 1989, Owen et al. 1989, Patton et al. 1989, Roop et al. 1985, Steele et al. 1985). Sandstedt and Ursing proposed the name *Campylobacter upsaliensis* for the "CNW group" and also provided a description of this species (1986, 1991).

Phenotypic characteristics

Cells of *C. upsaliensis* (Sandstedt and Ursing 1991) are described as microaerophilic, gram negative, oxidase-positive, non-spore forming, curved rods. Glucose is neither oxidized nor fermented. Catalase activity is either negative or weakly positive. Nitrates but not nitrites are reduced. Hippurate and urea are not hydrolyzed and no H_2S is produced in iron/metabisulphite medium or triple sugar iron agar. Most strains grow in 1% glycine and 1% bile and most are capable of anaerobic growth in the presence of 0.1% trimethylamine N-oxide hydrochloride. Growth in 1.5% and 3.5% NaCl does occur. Cells are motile by means of either a single polar flagellum or bipolar flagella. Cells can be S-shaped, gull-wing or helical and are 0.3-0.4 µm wide and 1.2-3 µm in length. Longer organisms with several helical turns have also been observed. Coccoid forms appear upon exposure of cells to air. Colonies are pinpoint, 1-2 mm in diameter, slightly raised, convex, translucent, and smooth. Growth occurs at 42°C but not 25°C. The optimal growth temperature is 37 °C. Growth at 30°C is variable. Cells are sensitive to nalidixic acid and cephalothin (Patton et al. 1989, Walmsley and Karmali, 1989). Preston et al. (1990) studied the susceptibility of 41 strains of "*Campylobacter upsaliensis*" to 24

antimicrobial agents and found that most strains are sensitive to the fluoroquinolones and beta-lactam antibiotics, but all strains were resistant to trimithoprim and teicoplanin.

Genotypic characteristics of *C. upsaliensis*

The genome size of *C. upsaliensis* (2,007 kb) is the largest of all the campylobacter genomes sized to date (Bourke et al. 1995). The first physical and genetic map of *C. upsaliensis* was constructed by Bourke et al. (1995). The position of ten genetic loci, including of that iron-uptake regulatory (*fur*) gene and flagellin A subunit (*flaA*) have been localized on the map. The genomic library of *C. upsaliensis* ATCC 43954 was constructed using a lambda Gem-11 vector. Some strains contain plasmid DNA but the function of the plasmids is not yet known (Owen and Hernandez 1990). The G+C content of this organism was determined to be 35-36 mol % (Sandstedt and Ursing 1991). The genotype, phenotype and ecology of *C. upsaliensis* has been found to be most closely related to *C. coli* and *C. jejuni* (Sandstedt et al. 1983, Ursing et al. 1983). Thompson et al. (1988) determined that *C. coli*, *C. upsaliensis*, *C. jejuni*, and C. *laridis* form a distinct rRNA subgroup.

Source, clinical relevance, and culture

C. upsaliensis has been isolated from the normal and diarrheic feces of dog and cat (Burnens and Nicolet 1992, Gebhart et al. 1984, Goossens and Butzler 1989, Goossens et al. 1990a, Goossens et al 1991, Goossens et al. 1990b, Hald and Madsen 1997, Hirschl et al. 1990, Moreno et al. 1993, Sandstedt et al. 1983, Stanley et al. 1994, Taylor et al. 1991), and from humans. In humans, *C. upsaliensis* can cause bacteremia (Babay et al.1996, Carnahan et al. 1994, Chusid et al. 1990, Hanna et al. 1994, Lastovica et al. 1989, Skirrow et al. 1993), miscarriage (Gurgan and Diker 1994), abscesses (Gaudreau and Lamothe 1992) and diarrhea (Lindblom et al. 1995, Megraud and Bonnet 1986, Taylor et al. 1989, Tee et al. 1987, Walmsley and Karmali 1989). Patton et al. (1989) observed the presence of *C. upsaliensis* in blood specimens obtained from patients suffering from gastroenteritis, ruptured ectopic pregnancy, fever and respiratory tract symptoms. Some of the patients were immunocompromised or suffered from chronic

diseases which perhaps increased their susceptibility to infection. Snijders et al. (1997) also found that the most frequently isolated species of the *Campylobacter* group in AIDS patients suffering from diarrhea was *C. upsaliensis*. Carter and Cimolai (1996) reported that Hemolytic-Uremic Syndrome (HUS) is associated with acute *C. upsaliensis* gastroenteritis. Sylvester et al. (1996) observed that *C. upsaliensis* can attach to epithelial cells due to the presence of specific lipid molecules that might serve as cell membrane receptors. *C. upsaliensis* was bound to phosphatidylethanolamine (PE), gangliotetraosylceramide (Gg4) and phosphatidylserine (PS). There was no binding to ceramide, cholesterol, phosphatidylcholine, and globosides. They also found that *C. upsaliensis* bound to purified human small-intestinal mucin, suggesting that these organisms also express an adhesin recognizing a specific mucin epitope. It has been speculated that binding to mucin might facilitate the binding of the bacteria to cell membrane receptors.

The media utilized to isolate *Campylobacter* species from clinical specimens contain various antibiotics that reduce contaminants. The most widely used medium was formulated by Skirrow (1977). Karmali et al. (1986) formulated a blood free, charcoal-based selective medium (CSM) consisting of Columbia agar base, activated charcoal (4 g/l), hematin (0.032 g/l), sodium pyruvate (0.1 g/l), cefoperazone (32 mg/l), vancomycin (20 mg/l) and cycloheximide (100 mg/l) for isolation of *C. jejuni* and *C. coli*. This medium can also be used for isolation of *C. upsaliensis* directly from stool samples without the need for filtration (Walmsley and Karmali 1989). Using another medium (CAT medium) containing cefoperazone (8 mg/l), amphotericin (10 mg/l) and teicoplanin (4 mg/l) (Aspinall et al 1993) recovery of *C. upsaliensis* can be increased 200-fold. The membrane filtration (0.65 µm pore size) of fecal specimens allows 90% recovery on CAT medium (Aspinall et al. 1996).

Coccoid forms of spiral organism

During a taxonomic study of the genus *Spirillum* Ehrenberg, Williams (1952) found that young cultures consisting entirely of spiral cells gradually changed until only

oval or spherical bodies were present in old cultures. Some *Vibrio* species can also transform to a coccoid morphology under certain conditions such as low temperature, as in *Vibrio vulnificus* (Oliver and Wanucha 1989, Nilsson et al. 1991, Weichart et al. 1992), or starvation, as in *Vibrio cholerae* (Hood et al. 1986). Another vibrioid bacterium which can form coccoid bodies is *Helicobacter pylori* (formerly known as *Campylobacter pylori*). Cellini et al. (1994) found that *H. pylori* developed a coccoid morphology within 20 days when grown microaerobically at 37°C in Brucella broth supplemented with 2% fetal calf serum. Moreover, the coccoid-like form of *H. pylori* can be induced by treatment with certain antibiotics such as erythromycin or other substances such as bismuth subcitrate, bismuth subsalicylate, or bile acids (Nilius et al. 1993).

Florent (1959) observed that cells of the genus *Campylobacter* can develop into the coccoid form. Karmali et al. (1981) observed that *Campylobacter jejuni* on blood agar plates incubated in air underwent rapid coccal transformation in 24 h and completely to coccoid form in 48 h, whereas normal morphology was maintained under microaerobic conditions. The storage of *C. jejuni* in air, particularly in the light, produces toxic factors in culture media which can affect the growth and shape of the cells (Bolton et al 1984, Fricker 1985, Hoffman et al. 1979, Juven and Rosenthal 1985). These toxic factors are probably reactive oxygen intermediates (Hoffman et al. 1979) such as superoxide anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide, which are formed as a result of the incomplete reduction of molecular oxygen (Juven and Rosenthal 1985). This is supported by the fact that the production of coccoid forms in suspensions stored in air was minimized if the culture medium was supplemented with superoxide dismutase which destroys superoxide radicals (Moran and Upton 1986). Ogg (1962) observed that the coccoid forms develop more slowly in broth and semisolid media than on solid media. A study of single colonies of *C. jejuni* on solid media by scanning electron microscope revealed that the periphery of colony consisted of the spiral forms, whereas the center and upper surface of the colonies, which received fewer nutrients than the periphery, consist of the coccoid forms (Lai-King et al. 1985).

Physiology of coccoid cells

Decline in viability of the VBNC form as in *Campylobacter* organisms and the possibility of resuscitation by animal passage, as first suggested by Rollins and Colwell (1986), is a highly debated topic in the literature. If coccoid cells can cause infection in animals they could be a public health threat and play a role in environmental contamination. Therefore, many investigations have focused on determining the physiological status as well as the infectious capacity of the coccoid form.

The cytoplasmic contents of the coccoid form have been studied in order to elucidate how and why cells undergo such dramatic morphological and physiological changes. The coccoid form of *Campylobacter jejuni* has been studied and found to contain less high molecular weight cytoplasmic components and nucleic acids than the vibrioid forms (Hazeleger et al. 1994, Moran and Upton 1986). Bodge et al. (1993) observed that the coccoid form of *H. pylori* contains polyphosphates as an energy and phosphorus source, which permits a certain level of endogenous metabolism to preserve RNA and DNA, as well as structural components like the cell wall and cell membrane for at least 3 months. However, Narikawa et al. (1997) found that the DNA and RNA contents of coccoid form of *H. pylori* were, respectively, 6.8- and 8.1-fold lower than those of helical *H. pylori*. Kusters et al. (1997) observed similar findings in another study. Jacob et al. (1993) observed no change in whole-cell protein and lipopolysaccharide patterns of coccoid and spiral-form cells of *C. coli* (which had converted under different conditions). Hazeleger et al. (1995) also observed that γ-radiation, which damages DNA, has no effect on the conversion of *C. jejuni*. Chloramphenicol which inhibits protein synthesis also has no effect on the conversion process (Boucher et al. 1994, Hazeleger et al. 1995). Therefore, it seems likely that synthesis of protein is not required for the transition of spiral to coccoid forms and that conversion is a passive process (Hazeleger et al. 1995). Coccoid forms have been found to contain different levels of certain enzymes. For example, Moran and Upton (1987a) found that the coccoid forms of *C. jejuni* contained lower amounts of superoxide dismutase than the spiral form. In addition, urease activity in *H. pylori* is lost when cells convert to the coccoid form (Nilius et al. 1993).

The energy status (i.e., ATP levels) of cells that transform into a coccoid form has also been studied. Beumer et al. (1992) observed that the ATP levels of coccoid suspensions of *C. jejuni*, which reflected the intracellular and extracellular ATP level, remain constant for a period of at least 3 weeks. During the first week of conversion, the intracellular ATP concentration actually increases. Hazeleger et al. (1995) compared the ratio between intracellular and extracellular ATP for coccoid conversion at different incubation temperatures (4°C, 12°C, and 25°C). Cells incubated at 4°C and 12°C showed comparable intracellular:extracellular ATP ratios (3.5 - 4), whereas cells converted at 25° C showed a significantly lower ratio (2) with more extracellular ATP present. This investigation showed that coccoid cells could maintain a certain amount of ATP although cells were not culturable (Hazeleger et al. 1995). Sörberg et al. (1996) studied the ATP levels of *H. pylori*. The *H. pylori* coccoid forms were not leaky as indicated by a lack of extracellular ATP in cell cultures. Addition of fresh media during the transition phase from the spiral to coccoid form of *H. pylori* also increased the intracellular ATP 26-fold. The coccoid form of *H. pylori* has 1000-fold lower ATP level per cell compared to the spiral form (Sörberg et al. 1996). Addition of fresh medium to the coccoid cultures from days 9 and 10 increased the ATP level twofold.

Cell wall integrity of the coccoid form has also been investigated. The gram stain reaction of coccoid forms compared with spiral forms of *C. jejuni*, investigated by electron microscopy, indicated that changes occurred in the integrity of the cell membrane during conversion (Moran and Upton 1986). Brock and Murray (1987) observed by electron microscope that the peptidoglycan layer of the coccoid form disappeared following conversion. The membrane fatty acids of the coccoid form of *C. jejuni* were investigated and found to be depended on temperature (Hazeleger et al. 1995). At 4°C and 12°C the membrane fatty acid composition of the coccoid form was nearly identical to that of spiral form, whereas it was very different at 25°C. At 25°C the membrane fatty acids (16:0 and 18:0) of coccoid form increased in concentration and 14:0 and 19:0 fatty acids decreased (Hazeleger et al 1995).

Campylobacter coccoid forms are generally nonmotile although flagella have been observed (Moran and Upton 1986), indicating that coccoid cells may be a degenerative

stage unable to supply the flagella with sufficient energy for motility or that the flagellar apparatus itself is degenerate (Moran and Upton 1987b). Kusters et al. (1997) observed that *H. pylori* cells in the coccoid form lacked a membrane potential. There is also evidence that coccoid forms undergo several antigenic changes. For example, Cole et al. (1997) observed that coccoid forms of *H. pylori* bound poorly to gastric epithelial cells and induced low, barely detectable, levels of interleukin-8 secretion when compared to the spiral form.

Relationship between viable but non-culturable (VBNC) state and the coccoid forms

Colwell et al. (1985) introduced the term "viable but non-culturable calls" to describe particular cells which appeared in populations of some gram-negative bacteria (*E. coli, Salmonella and Vibrio* spp.) that had been starved in aquatic environments. In an aquatic environment, pathogenic bacteria, which were capable of growing in warm-blooded hosts, may be stressed by starvation, exposure to toxic chemicals, suboptimal temperature or saline conditions. As a consequence, these cells may enter a viable but nonculturable stage as a survival strategy until environmental conditions change (Kjelleberg et al. 1987). Oliver (1993) defined VBNC cells as bacteria that are metabolically active yet incapable of undergoing cellular division required for growth. VBNC cells are usually smaller than cells from which they are produced (Roszak and Colwell 1987a); however, in the case of *E. coli*, sometimes the cells do not change size and appear intact (Barcina et al. 1989, Reeve et al. 1984). Viability of VBNC forms has been estimated microscopically by using fluorochrome stains (Hobbie et al 1983), microradioautography (Shahamat et al. 1993), and viability testing with tetrazolium salts (Kaprelyants and Kell 1993, Maki and Remsen 1981, Oliver and Wunucha 1989, Zimmermann et al. 1987).

Not all bacteria undergo a morphological change to cocci upon conversion to the VBNC state. Examples of species that do not undergo morphological changes include *Yersinia ruckeri* (Romalde et al.1994), *Pseudomonas fluorescens* DF 57 (Binnerup et al. 1993), *Aeromonas salmonicida* (Morgan et al. 1993), *Shigella sonnei* (Colwell et al. 1985), *Shigella dysenteriae* (Islam et al. 1993), *Legionella pneumophila* (Bej et al. 1991,

Hussong et al. 1987), *Escherichia coli* (Granai and Sjogren 1981), *Salmonella enteritidis* (Roszak et al. 1984).

Resuscitation of VBNC coccoid forms to culturable spiral form?

There are many examples in which VBNC cells of some species have been recovered on solid media after the application of various resuscitation procedures. For example, Nilsson et al. (1991) resuscitated starved *Vibrio vulnificus* cells by exposing the cells to a shift in temperature. In this study, *Vibrio vulnificus* cells were starved at 5°C in which a time-dependent loss in viability was observed on agar plates. The viability (culturability) was completely restored by incubation of the culture at room temperature in saline (0.85 % NaCl). Transitions from rods (vegetative cells) to small cocci (the end of starvation) and vice versa (during resuscitation) have been observed.

Recently, there have been claims of resuscitation of certain microaerophilic spiral organisms by animal passage. For example, Cellini et al. (1994) found that coccoid *H. pylori* could be recovered in BALB/c mice stomachs after 2 weeks of inoculation. Saha et al. (1991) also found that coccoid *C. jejuni* cells could be recovered in a rat gut and Jones et al. (1991) acheived some recovery in the gut of suckling mice. In contrast, Medeman et al. (1992) failed to recover the VBNC coccoid form of *C. jejuni* in 1-day-old chicks and Enroth and Engstrand (1996) failed to demonstrate the growth of the coccoid form of *H. pylori* following egg passage. Whether or not “true” recovery really occurred is debatable because of the possibilty that spiral forms could have contaminated the coccoid forms used for animal passage. If this is the case, any growth observed would be a result of contamination. In addition, even though direct plate counts of a portion of the inoculum fed to the animals was done in those experiments and no viable growth was observed, the rest of the coccoid form preparation that was used for animal passage may have been contaminated by only one or a few spiral forms that were not readily seen by direct microscopic count.

Peptidoglycan

The essential cell wall polymer of most eubacteria for maintaining the shape and size of bacterial cells is peptidoglycan (synonym: murein or murein sacculi). The murein sacculus allows *E. coli* cells to withstand high turgor pressure and endows the bacterium with mechanical strength, maintaining the rod shape of *E. coli* (Höltje and Schwarz 1985, Nanninga 1988, Weidel and Pelzer 1964). Cover et al. (1991) observed that when *E. coli* was treated briefly with polymyxin B to create pores in the outer membrane and allow the penetration of lysozyme, the cells changed from a rod to a coccoid shape after its peptidoglycan was digested; moreover, the coccoid forms were osmotically stable, unlike spheroplasts prepared by ordinary procedures in which the integrity of the outer membrane is severelky compromised. The peptidoglycan of gram positive bacteria is multilayered; whereas, the peptidoglycan of gram-negative bacteria is a monolayer at lateral or cylindrical wall and triple-layer at the polar caps or septa of cell (Signoretto et al. 1996).

The peptidoglycan is a heteropolymer consisting of glycan strands crosslinked by peptides (Fig. 1). The glycan strands are repeating disaccharide units, consisting of *N*-acetylmuramic acid and *N*-acetylglucosamine joined together in an alternating sequence by (β-1$\rightarrow$4)-glycosidic bonds. The glycan linkages of peptidoglycan are considered to be uniform in all bacteria with every *D*-lactyl group of the *N*-acetylmuramic acid being peptide-substituted. All glycans have short tetrapeptide units terminating with *D*-alanine or tripeptide units lacking the terminal *D*-alanine. The *L*-alanine at the *N*-terminus can be replaced by *L*-serine or glycine in some species.

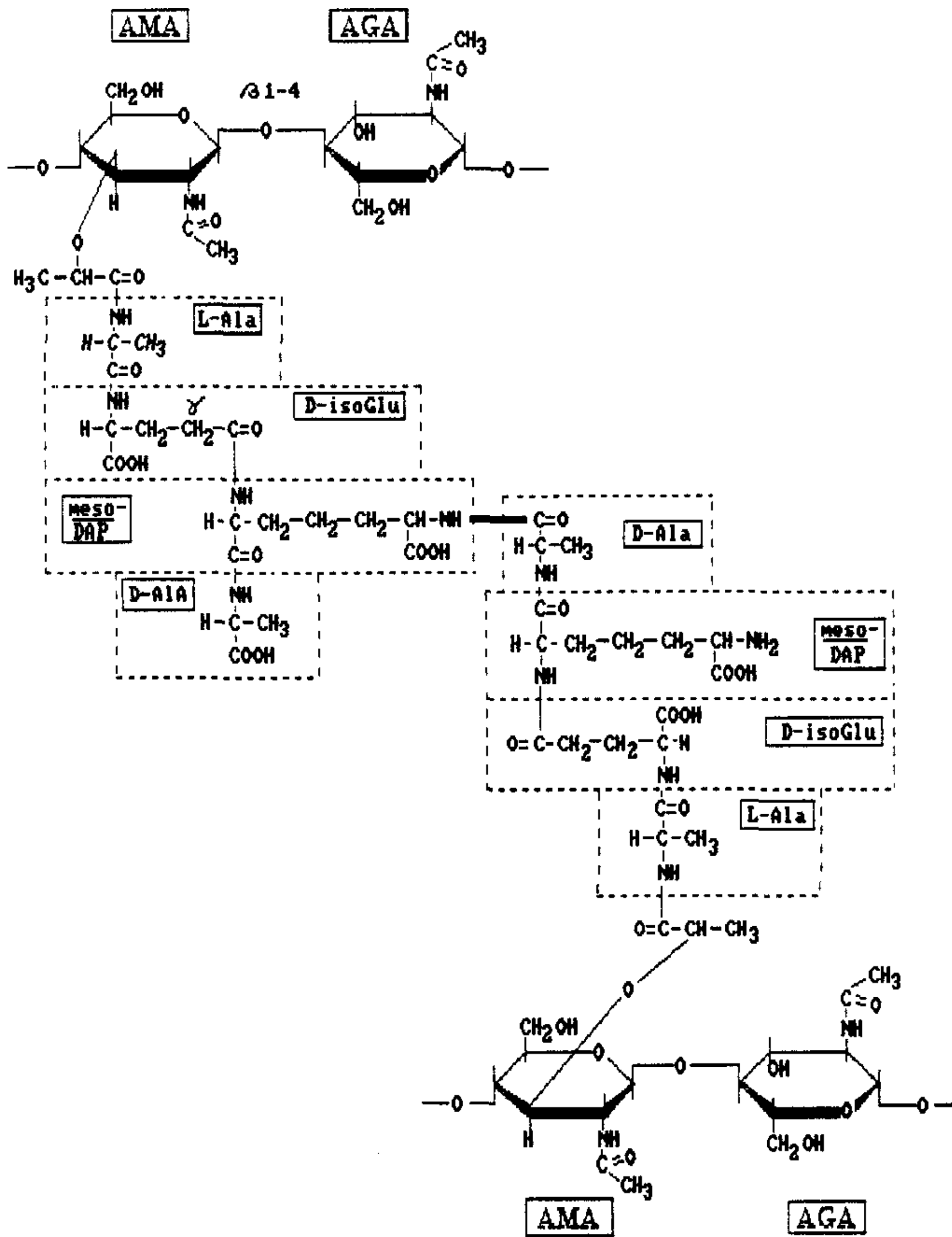

Figure 1

Crosslinkage of the glycans is achieved by peptides linked to the lactyl group of the muramic acid moiety. There are four main types of interpeptide bridges linking peptidoglycans:

Type I. A direct peptide bond between *D*-alanine and the diaminoacid at position 3. In the case of gram-negative bacteria, amino acid #3 is nearlyl always *meso*-diaminopimelic acid (DAP).

Type II. A bridge between *D*-alanine and the diaminoacid, such as pentaglycine or other *L*- or *D*-amino acid sequences (see Table 1). Bridges occur only in gram-positive bacteria.

Table 1. Types of interpeptide bridges in addition to pentaglycine in various bacteria

Amino acid(s)	Organism
-[Gly]$_5$-	*Staphylococcus aureus*
-[*L*-Ala]$_3$-*L*-Thr-	*Micrococcus roseus*
-[Gly]$_5$-[*L*-Ser]$_2$-	*Staphylococcus epidermidis*
- *L*-Ser- *L*-Ala-	*Lactobacillus viridescens*
- *L*-Ala- *L*-Ala-	*Streptococcus pyogenes*
- *L*-Ala-	*Arthrobacter crystallopoietes*
	Enterococcus hirae
-*D*-Asp-NH_2-	*Enterococcus faecium*
	Enterococcus hirae
	Lactobacillus casei

Type III. In gram-positive bacteria, a bridge composed of one to several peptides, each having the same amino acid sequence as the peptide unit attached to muramic acid.

Type IV. In gram-positive bacteria, a bridge extending between the –COOH group belonging to *D*-glutamic acid in position #2 and the terminal D-alanine. Such a bridge must contain at least one diamino acid.

The peptidoglycan of *C. jejuni*, *C. coli*, and *C. fetus* is composed of muramic acid, glucosamine, alanine, glutamic acid, and diaminopimelic acid in a molar ratio of 1.1 **:** 1 **:** 1.7 **:** 1.1 **:** 0.9 (Amano and Shibata 1992) and has the same composition as that in *E. coli*. Thirty percent of the diaminopimelic acid is cross-linked by peptides compared to 50 % in *E. coli* and *Selenomonas ruminantium.* It is possible that the low degree of cross-linkage of amino groups in *Campylobacter* peptidoglycan makes it easier to transform the spiral forms of this genus to the coccoid forms.

Synthesis of the extracellular macromolecule murien is initiated by the formation of UDP-activated *N*-acetylmuramyl pentapeptide in the cytoplasm (Rogers et al. 1980). To facilitate translocation of this hydrophilic precursor across the membrane, it is linked to a lipid carrier molecule, undecaprenyl phosphate (bactoprenol). This lipid-bound intermediate is further modified with *N*-acetylglucosamine prior to its export to the periplasmic surface. The disaccharide pentapeptide unit is then added to the existing murein by membrane-bound enzymes (murein synthetases) that crosslink the incoming new subunits in two directions.

Autolysins

A wide variety of bacteria have been found to possess murein (peptidoglycan) hydrolases, many of which are capable of dissolving the cells and/or walls of the producing strain. Therefore, they are called autolysins. Autolysins have been found so frequently that they are believed to occur in all bacteria. Autolysins are defined as endogenous enzymes that hydrolyze specific bonds in the bacterial cell wall (peptidoglycan) resulting in damage to the integrity and protective properties of the two- or three-dimensional structure of the peptidoglycan (Shockman and Barrett 1983). This definition does not include every enzyme that is capable of hydrolyzing bonds in the peptidoglycan. Included in the term

peptidoglycan hydrolases but excluded from the definition of autolysins would be those enzymes that can hydrolyze bonds that are not directly involved in providing stability of the peptidoglycan sacculus, such as *DD*-carboxypeptidases that remove residual *D*-alanines that are not involved in crosslinking. Autolysins can belong to one of two basic types, glycosidases and peptidases (Höltje and Shockman 1994, Höltje 1995), as follows:

1. Glycosidases (Glycan strand hydrolyzing):

a. β-*N*-acetylmuramidases (muramidases or lysozymes): capable of hydrolyzing the β-1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine.

b. β-*N*-acetylglucosaminidases: capable of hydrolyzing the β-1,4-glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid.

c. Lytic transglycosidases: capable of splitting the β-glycosidic bond and concomitantly catalyzing the transfer of the glycosyl bond onto the hydroxyl group of the 6th carbon of the same muramic acid, thereby forming (1→6)-anhydromuramic acid.

2. Peptidases:

a. Endopeptidases are capable of dissolving the murein network by cleaving peptide crossbridges. Three different murein *DD*-endopeptidases that hydrolyze the *DD*-peptide bond between *D*-alanine and meso-diaminopimelic acid have been characterized in *E. coli*. Two of them are penicillin-sensitive enzymes and belong to the family of penicillin-binding proteins (PBPs) as PBP4 and PBP7. The third DD-endopeptidase is a penicillin-insensitive enzyme named MepA.

b. *N*-acetylmuramoyl-L-alanine amidases (amidases): specifically cleave the amide bond between the lactyl group of muramic acid and the α-amino group of L-alanine, the first amino acid of the stem peptide.

Autolysin assay

Fleming's classical assay (1922) for hydrolases or autolysins is nonspecific and is based on observing the dissolution of *Micrococcus luteus* (formerly *Micrococcus* lysodeikticus) cells, usually by measuring a decrease in turbidity of the cell suspension. More specific assays which are similar to Fleming's classical assay measures the decrease in turbidity of insoluble cell wall or peptidoglycan substrates. Ghuysen et al. (1966) developed assays using similar subtrates that measured the release of amino- or carboxy-terminal and/or reducing groups. The specificity of the bond hydrolyzed can be determined by identification of the released terminus. For example, the action of an amidase results in an increase in *N*-terminal *L*-alanine, whereas the action of a muramidase or glucosaminidase results in an increase in reducing *N*-acetylmuramic acid or *N*-acetylglucosamine, respectively. More sensitive assays involve adding autolysins to specifically radioactive-labeled peptidoglycan and determining the amount of radioactivity in the supernatant after separation of the insoluble peptidoglycan by centrifugation (Hartmann et al. 1972, Shockman et al. 1967).

Recently, the detection and separation of autolysins have been achieved by zymogram analysis, i.e., by incorporating a turbid substrate into a SDS-polyacrylamide gel before electrophoresis and then renaturing the SDS-denatured enzymes after electrophoresis. Autolysins are detected in the areas of the gel in which zones of clearing occur. This technique has been used to study the autolysins of *Bacillus* spp. by embedding whole cells of *M. luteus* in the SDS-PAGE gel and removing the SDS (allowing enzymes to renature) with low ionic strength Tris-HCl buffer (Potvin et al 1988). Purified peptidoglycan from gram positive bacteria has also been used as a substrate (Laclerc and Asselin 1989). Gram positive bacteria have thick cell walls containing many layers of peptidoglycan, and the purified peptidoglycan yields a suitable turbidity when incorporated into gels. The peptidoglycan from gram negative bacteria, however, being much thinner, provides less turbidity and does not serve well as a turbid substrate (Laclerc and Asselin 1989). However, Watt and Clarke (1994) developed a staining method using methylene blue for gels containing peptidoglycan from gram negative bacteria that allows lytic bands to become readily visible as colorless areas against a blue background.

As noted by Foster (1992), the renaturing gel technique has limitations. It is unlikely that all of the autolysins of an organism can be detected by this method. The methodology requires that an autolysin (1) survives SDS-PAGE, (2) can be renatured to an enzymatically active form after SDS-PAGE, (3) is active in monomeric form or composed of identical subunits that can reassociate, (4) hydrolyzes enough bonds to cause visible dissolution of the substrate, (5) hydrolyzes and dissolves the substrate in the gel (the substrate specificity problem), and (6) does not require cofactors or activators such as processing by the action of a protease.

HPLC-based separation of peptidoglycan subunits (Glauner 1988), specific enzyme assays for various peptidoglycan hydrolases including carboxypeptidases, endopeptidases (Glauner et al. 1988) and amidases (Harz et al. 1990) have also been established.

Physiological function of autolysins in bacteria

The presence of autolysins in cultures of rapidly dividing bacteria led to the idea that such potentially suicidal enzymes might play one or more roles in cell wall assembly and bacterial growth (Shockman 1965, Shockman et al. 1958,Weidel and Pelzer 1964). In several bacterial species the capacity of bacterial cells to autolyze was maximal or near maximal during the exponential growth phase (Coyette and Shockman 1973S, Mitchell and Moyle 1957, Shockman et al. 1958, Young 1966). The potential roles of autolysins in cell growth and cell division have been supported by various experiments (Daneo-Moore and Shockman 1977, Rogers 1970, Roger et al. 1980). These roles include :

1. Growth of microbial cells

Peptidoglycan hydrolases of only two specificities could provide new acceptor sites for addition of new wall material:: *N*-acetylmuramidases (including the lytic transglycosylases) and appropriate endopeptidases (Daneo-Moore and Shockman 1977, Rogers 1970, Roger et al. 1980). This is consistent with current knowledge of cell wall biosynthesis (Shockman and J.-V. Höltje 1994). New peptidoglycan could be attached to the new acceptor site with the help of other hydrolases.

2. Cell division and separation.

It seems likely that hydrolysis of some bonds in the peptidoglycan are involved in the separation of the two new cell poles. Correlation between deficiencies in autolytic activity and failure of cells to separate have been found in several coccal and rod-shaped species (Chatterjee et al. 1969, Fan and Beckman 1973, Forsberg and Rogers 1971, Pooley et al. 1972, Shungu et al. 1979, Tomasz 1968, Wolf-Watz and Normark 1976).

3. Peptidoglycan turnover

Cell wall turnover is defined as the loss of previously assembled, insoluble wall from the bacterial cells. Due to a lack of an outer membrane in gram positive bacteria, the turnover peptidoglycan can be measured in the supernate of culture media. Loss of wall and wall peptidoglycan has been observed during growth of *Bacillus subtilis* (Doyle and Koch 1987), *Lactobacillus acidophilus*, *Staphylococcus aureus, Listeria monocytogenase, Enterococcus hirae* (Boothby et al. 1973), *Streptococcus sanguis* (Mychajlonka et al. 1980) and a Lys^- DAP^- mutant of *Bacillus megaterium* (Pitel and Gilvarg 1970). Loss of peptidoglycan in gram negative bacteria is not as easily assayed due to the presence of an outer membrane. The release of peptidoglycan fragments from the peptidoglycan sacculus of *E. coli* has been observed (Goodell and Schwarz 1985; Doyle et al. 1988). It is believed that these fragments may be re-utilized for peptidoglycan assembly (Goodell 1985, Goodell and Higgins 1987, Park 1993) There are many different types of autolysins involved in peptidoglycan turnover. For example, a muramidase of *L. acidophilus* (Coyette and Ghuysen 1970) and lytic transglycosylases and endopeptidases in *E. coli* are responsible for peptidoglycan turnover. Amidases and glucosaminidases are involved in further hydrolysis of the turnover products creating smaller units that can be reutilized for peptidoglycan assembly following uptake into the cytoplasm via an active transport system (Goodell and Higgins 1987).

4. Cell transformation

Lacks and Neuberger (1975) reported that protoplasts of pneumococci are caused by the action of autolytic enzymes. Seto and Tomasz (1975) found that competence factor (CF) added to streptococci increased the rate at which pneumococci lysed. They proposed that CF affected membrane permeability and that this change allowed exit of autolytic enzymes from the cytoplasm and access to the cell wall. It was also suggested that autolytic activity had a role in the transformation of streptococci since the addition of autolysin inhibitors such as *N*-ethylmaleimide or mercuric chloride inhibited both autolysis and the development of competence. Amano and Shibata (1992) found that the amount of peptidoglycan of the coccoid form of *C. jejuni* and *C. coli* is low when compared to those of spiral form. They suggested that the transformation from the spiral to coccoid form of *C. coli* and *C. jejuni* is accompanied by an enzymatic degradation of peptidoglycan.

5. Spore formation and germination

Examination of sporulating cultures of *Bacillus subtilis* and *B. sphaericus* (Guinand et al. 1974, Guinand et al. 1976, Guinand et al. 1978, Guinand et al. 1979) shows the presence of endopeptidase and amidase. Endopeptidase hydrolyzes peptide bonds in the peptidoglycan. In *B. thuringiensis* the bond between *L*-alanine and *D*-glutamate is hydrolyzed whereas in *B. subtilis* and *B. sphaericus* the bond between γ-D-glutamyl and the meso-diaminopimelic acid residues (Guinand et al. 1974) is hydrolyzed. It is likely that the endopeptidase is specific to the developmental process. It was also discovered that endopeptidases are not present in vegetative cells but do appear during sporulation. This enzyme activity is depressed if sporulation is inhibited by netropsin, a compound that inhibits RNA synthesis (Guinand et al. 1976, Guinand et al. 1978). For spore germination, Brown and Cuhel (1975) reported that the surface–localized cortex-lytic enzyme in the spores of *B.*

cereus can be extracted and the extracted enzyme can solubilize the fragments of spore integument. The enzyme was purified and shown to be an endo-β-*N*-acetylhexosaminidase (Gould et al. 1966).

Localization of autolysins

Autolysins must be transported across the cytoplasmic membrane in order to effect the peptidoglycan substrate. It is believed that autolysins either remain attached or anchored to the outer surface of the cytoplasmic membrane or they bond directly to the peptidoglycan (Shockman and Höltje 1994). The binding of hydrolases to peptidoglycan has been found to be quite strong. In order to release the autolysins from peptidoglycan, salt concentrations up to 5-8 M LiCl (Pooley et al. 1970), 0.01 N NaOH (Coyette and Shockman 1973), or 4-8 M guanidine-HCl (Kariyama and Shockman 1992) must be used. In addition, it has been found that a soluble lytic transglycosylase of *E. coli* remains bound to the murein sacculus even after boiling in 4% sodium dodecylsulfate (Walderich and Hö ltje 1991). Using an electron microscope, amidases of *B. subtilis* (Hobot and Rogers 1991) and *Streptococcus pneumoniae* (Díaz et al. (1989) have been found to bind to sites of cell division (in the septa of dividing cells). The soluble lytic transglycosylase of *E. coli* has been shown to bind exclusively to the outer surface of the peptidoglycan sacculus. The 26 kDa autolysin of *Pseudomonas aeruginosa* was found mainly in periplasm by using immunogold-labeling technique (Li et al. 1996).

Regulation of autolysins

Regulation of autolysin activity might occur at several levels (Shockman and Hö ltje 1994).

1. Autolysins are synthesized on cytoplasmic ribosomes and are transported through the cytoplasmic membrane to the exterior wall of gram positive bacteria or to the periplasm of gram negative bacteria, providing access to the substrate. Thus, control of general access to the insoluble substrate is an important factor. Some autolysins require proteolytic activation (Conover et al.

1966, Pooley and Shockman 1969, Shockman et al. 1967) and this may be another important factor in regulation. The mechanism of protein folding (or refolding) could also be an important factor in the activity of the muramidase-2 of *Enterococci hirae* (Kariyama and Shockman 1992).

2. The ability of autolysins to bind to and maintain contact with the insoluble substrate outside the cell barrier could also be an important limiting factor. Several autolysins have been shown to bind their substrate with very high affinity, and, in at least two instances, shown to bind to a limited number of sites (Kariyama and Shockman 1992, Walderich and Höltje 1991). The muramidase-2 of *E. hirae* (Chu et al. 1992) and the autolysin of *Streptococcus faecalis* (Beliveau et al. 1991), as well as the amidase and bacteriophage-coded amidase and glycosidase of pneumococci (García et al. 1988), possess long repeated amino acid sequences that could be involved with substrate binding.

3. Substrate specificity is also important. The change in the chemistry of the peptidoglycan might control the action of the autolysins. *O*-acetylation of amino sugars of the peptidoglycan has been shown to decrease the susceptibility to hydrolysis by hen egg white lysozyme (Clarke 1993, Payie et al. 1996, Dupont and Clarke 1990, Rosenthal et al. 1982). Wall polymers other than the peptidoglycan substrate, such as polysaccharides and teichoic acids, also appear to be important for autolysin binding and could, therefore, affect substrate specificity. For example, the choline-containing teichoic acid of pneumococcus appears to be important in the binding of the pneumococcal amidase to the cell wall (Giudicelli and Tomasz 1984). Cell walls devoid of teichoic acid have lower affinity than normal cell walls (Herbold and Glaser 1975a, Herbold and Glaser 1975b). Acetylated lipoteichoic acid (LTA) also inhibits autolysis of walls (Cleveland et al. 1976a); however, the lipoteichoic acid is less inhibitory than phospholipid of the wall (Cleveland et al. 1976b).

Höltje and Tomasz (1975) found that choline-containing pneumococcal lipoteichoic acid (Forssman antigen) is a powerful inhibitor of *N*-acetylmuramyl-L-alanine amidase.

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Chapter 1

A Study of the Coccoid Form of *Campylobacter upsaliensis*

Abstract

Conversion of *Campylobacter upsaliensis* to the nonculturable but viable coccoid form was characterized. Chloramphenicol did not prevent the conversion. Severe decreases in isocitrate dehydrogenase activity and oxygen uptake and extensive degradation of ribosomal RNA suggest that the coccoid form is a degenerative form rather than part of a life cycle.

Campylobacter upsaliensis is a microaerophilic, vibrioid bacterium found in the normal and diarrheic feces of dogs and cats (Sandstedt and Ursing 1991, Burnens and Nicolet 1992) and in humans it can cause bacteremia (Lastovica 1989, Chusid et al. 1990, Carnahan et al. 1994, Babay et al. 1996), miscarriage (Gurgan and Diker 1994); abscesses (Gaudreau and Lamothe 1992), and diarrhea (Megraud and Bonnet 1986, Lindblom et al. 1995). It is closely related to *C. coli* and *C. jejuni* in genotype, phenotype and ecology (Sandstedt et al. 1983) and changes to a coccoid morphology when exposed to air (Sandstedt and Ursing 1991). The coccoid forms of *Campylobacter jejuni* (Moran and Upton 1986, Jone et al. 1991, Saha et al. 1991, Sandstedt and Ursing 1991) and *Campylobacter coli* (Jacob et al. 1993) have been reported to be viable but not culturable (VBNC) but this is not known for *C. upsaliensis*. This report assesses the culturability of the coccoid forms of this species and compares the oxygen uptake, enzyme activity, and condition of the ribosomal RNA (rRNA) in the vibrioid vs. the coccoid form.

Campylobacter upsaliensis VPI strain CG-1 was obtained from C. Gebhart, Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, MN and maintained in semisolid Brucella agar (Difco). Log phase cultures were obtained by inoculating 2 l of Brucella broth with 20 ml of a 48-h-old culture grown in Brucella broth and incubating at 37°C under an atmosphere of 6% O_2, 3% CO_2 and 91% N_2 in a reciprocal shaking waterbath at 40-45 oscillations per min. Mid-log phase occurred in 22-24 h at 80-84 nephelometer turbidity units as measured with a ratio turbidmeter (Hach Co., Loveland, Colo, USA)..

For determining the optimum temperature for conversion to the coccoid form, 50-ml portions of a mid-log phase culture were transferred to four sterile 250-ml flasks for static aerobic incubation at varioius temperatures. At 30 and 37°C conversion occurred in 4 d whereas at 25°C it took 7 d (Table 1). Maximum conversion was 95% and never reached 100% even with incubation up to 30 d. At 4°C only 10% of the vibrioid forms converted in 3 d and no further increase occurred with prolonged incubation. Two repetitions of the experiment gave similar results and 37°C was chosen for all conversion experiments. Although the minimum bactericidal concentration of chloramphenicol for mid-log phase cultures was 16 µg per ml, 128 µg per ml failed to prevent conversion of

mid-log phase cultures to the coccoid form. Boucher et al. (1994) and Hazeleger et al. (1995) reported similar results for *Campylobacter jejuni* and proposed that conversion did not require protein synthesis.

The spread plate method was used to enumerate CFUs in midlog cultures and cultures subsequently exposed to air. Brucella agar plates (Difco) were incubated under an atmosphere of 6% O_2, 3% CO_2, and 91% N_2. On the first day of air exposure colony counts decreased more than 99.99% compared to the midlog phase and from the second day to the fifth day of air exposure no colonies developed (Table 2). For vital staining of cells, 20-µL portions of cultures were added to 2 ml of 0.1% acridine orange (AO) for 2 min. The mixture was filtered through a black membrane filter (0.22 µm pore size; Micron Separations Inc., Westborough, MA) and the cells were observed by epifluorescence microscopy. For all of the cultures, >95% of the cells— both vibrioid and coccoid—exhibited orange-red fluorescence (Table 2).

Preliminary studies indicated that mid-log phase cells contained cytoplasmic isocitrate dehydrogenase (ICDH; EC 1.1.1.42) of high specific activity. To compare the activity in the vibrioid form vs. the coccoid form, 2-ml samples were removed from cultures at various times of exposure of the batch culture to air, centrifuged at 16,000 × *g*, washed once with phosphate-buffered saline (pH 7.4), and disrupted by sonic oscillation. Whole cells and large particles were removed at 16,000 × *g* and the supernatant was assayed for ICDH activity by the method of Daron (1966) modified by decreasing the concentration of the $MnCl_2$ to 1 mM to prevent turbidity and by increasing the concentration of the DL-sodium isocitrate to 50 mM to obtain maximal activity. The increase in absorbance at 340 nm was linear with time and protein concentration. Total protein was determined by the assay method of Bradford (1976). The specific activity of ICDH decreased by 40% on the first day of air exposure compared to exponential growth phase cells, by 85% on the second day, and by >95% on the third day to the fifth day (Table 3).

For measurement of oxygen uptake, the spiral form from a mid-log phase culture and the coccoid form from a 4-day-old culture were washed twice with 40 mM Tris-HCl buffer (pH 7.0) and suspended in 20 ml of this buffer. The protein content of the whole

cells was determined by the assay method of Bradford (1976). Oxygen consumption was measured with an oxygen monitor calibrated by the method of Robinson and Cooper (1970). The oxygen electrode was inserted into a water-jacketed Clark cell-type chamber (Gilson Medical Electronics, Middleton, WI 53562) maintained at 37°C. Electron donors were injected into the cell suspension to a final concentration of 10 mM (formate was used at 0.05 mM). Oxygen uptake by the vibrioid form with succinate, malate, and formate was 126, 3200, and 3600 nanomoles O_2 per min per mg protein, respectively, whereas no detectable oxygen uptake occurred with the coccoid form. Neither form exhibited detectable oxygen uptake with fumarate, isocitrate, glutamate, aspartate, alanine, glucose, fructose, galactose, and ribose.

For characterization of rRNA, *C. upsaliensis* was cultured in six 200-mL volumes of Brucella broth at 37°C, with a gas mixture of 6% O_2, 3% CO_2, and 94% N_2 bubbled continuously through the medium. At mid-log phase one culture was centrifuged at 7500 $\times$ *g* for 10 min and the cells were suspended in 2 ml of suspending buffer containing dithiothreitol (Johnson 1994). Total RNA was isolated by the guanidine isothiocyanate isolation procedure and subjected to agarose gel electrophoresis as described by Johnson (1994). The remaining cultures were incubated statically under an air atmosphere for various periods before extracting the RNA. Cells in the exponential growth phase showed intact 23S, 16S, and 5S rRNA but on the first day of air exposure the cells began showing apparent degradation of 23S and 16S (Fig. 1). One fragment occurred below the 23S band and two fragments below the 16S band, suggesting preferential sites of attack. By the second day to fifth day of air exposure the RNA showed more extensive degradation with nearly complete disappearance of the 23S band. Two repetitions of the experiment yielded similar results.

Whether or not the vegetative form of *C. upsaliensis* can be resuscitated from the coccoid form cannot be determined definitively from these experiments. Some investigators (Jones et al. 1991; Saha et al. 1991) have reported success in resuscitating the viable-but-nonculturable coccoid form of *C. jejuni* by animal feeding experiments but others have not (Medema et al. 1992). Such experiments are open to the criticism that a few vegetative forms may have been present in the portion fed to the animal and that these

may have multiplied, leading to the erroneous conclusion that that resuscitation had occurred. In the case of *C. upsaliensis* strain CG-1, although no growth occurred on Brucella agar by the second day of air exposure, vital staining with AO suggested that most of the coccoid cells might still be viable. AO fluoresces green when attached monomerically to double-stranded DNA and orange-red when attached as a dimer to single-stranded RNA or denatured DNA (Daley 1979). Viable cells fluoresce orange-red because they contain much more RNA than DNA whereas nonviable cells contain little RNA. Since most of the RNA in bacterial cells is rRNA, this suggests that the coccoid cells, although nonculturable on Brucella agar, may still possess the ability to synthesize proteins and thus might be able to be resuscitated under suitable conditions. However, degenerative forms of rRNA, as indicated by a smear on agarose gel electrophoresis, also can bind to fluorochrome vital stains (Yamamoto et al. 1996). Yamamoto et al. (1996) reported that electrophoresis of nucleic acids extracted from nonculturable cells of *Legionella pneumophila* revealed degradation of 23S, 16S, and 5S rRNA. Tolker-Nielsen and Molin (1996) have suggested that the degradation of rRNA is a direct cause of bacterial cell death. Other evidence to support a lack of viability in *C. upsaliensis* includes the lack of isocitrate dehydrogenase (ICDH) activity and lack of detectable oxygen uptake. All of these results taken together suggest that the coccoid form of *C. upsaliensis* is a degenerative form rather than a dormant form that is part of a life cycle.

TABLE 1. Effect of incubation temperature on conversion of the vibrioid form of *C. upsaliensis* to the coccoid form

Temperature (°C)	Incubation time (d)	Percent coccoid forms[a]
4	3	10[b]
25	7	>95
30	4	>95
37	4	>95

[a] By phase-contrast microscopy.

[b] No further increase in percent coccoid forms occurred with prolonged incubation.

TABLE 2. Viability measurements of *C. upsaliensis* CG-1 by colony counts and acridine orange staining

Time after exposure to air, h	CFU per ml[a]	% Fluorescing orange-red by acridine orange stain
0[b]	$1.8 \pm 0.3 \times 10^{10}$	100
24	$1.5 \pm 0.2 \times 10^{5}$	99
48	NG[c]	98
72	NG	95
96	NG	95
120	NG	95

[a] Values represent the mean and standard deviation from three different experiments.

[b] Cells were from the exponential phase of growth.

[c] NG = no growth.

TABLE 3. Isocitrate dehydrogenase (ICDH) activity in *C. upsaliensis* CG-1 after various periods of air exposure

Time after exposure to air, h	Specific activity (µmole of substrate min^{-1} mg $protein^{-1}$)
0[a]	514 ± 8[b]
24	290 ± 4
48	77 ± 4
72	27 ± 2
96	18 ± 2
120	3 ± 1

[a] Cells were from the exponential phase of growth.

[b] Values represent the mean and standard deviation from three different experiments.

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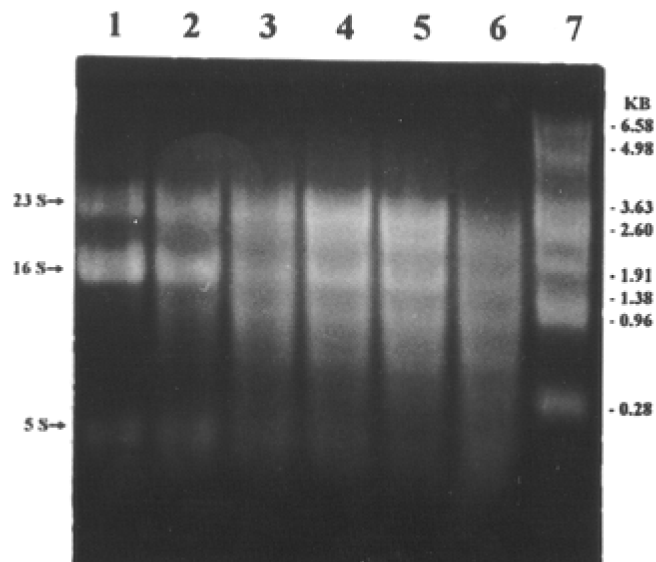

FIG. 1. Agarose gel electrophoresis of rRNA isolated from *C. upsaliensis* during exponential growth (lane 1) and at 1, 2, 3, 4 and 5 days of exposure of the culture to air (lanes 2-6, respectively).

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Chapter 2

Detection of a *Campylobacter upsaliensis* autolysin

Abstract

Autolytic activity in the soluble and sediment fractions of sonicates of the spiral and the coccoid form of *Campylobacter upsaliensis* could not be demonstrated by native (nondenaturing) PAGE. Autolysins were detected, however, by using denaturing SDS-PAGE gels containing either purified *E. coli* peptidoglycan or whole cells of *Micrococcus luteus* as the turbid substrate, with subsequent renaturation by treatment with Triton X-100 buffer. In renaturing gels that contained *E. coli* peptidoglycan, 14 autolytic bands were detected ranging from 200 kDa to 12 kDa. In similar gels containing whole cells of *M. luteus,* only a single band appeared having a molecular weight of 34 kDa. This band corresponded to one of the bands present in the gels containing *E. coli* peptidoglycan. This common autolysin was isolated by adsorbing it from *C. upsaliensis* lysates onto *M. luteus* cells and then subjecting these cells to renaturing SDS-PAGE in gels containing *E. coli* peptidoglycan. The 34 kDa autolysin differed from a single 51 kDa autolysin unique to the *M. luteus* cells and when isolated from an SDS-PAGE gel was pure when tested by isoelectric focusing. The N-terminal amino acid sequence analysis showed the first 15 amino acids of the 34 kDa autolysin to have 67% identity to a part of antigenic protein PEB4 of *Campylobacter jejuni*. The purified autolysin was used to immunize rabbits and the antibodies produced precipitated autolytic activity from cell lysates. The specificity of the antibodies was shown by Western blotting: only a single specific band occurred, with a molecular weight of 34 kDa, and thus it seems unlikely that the 34 kDa autolysin was derived from any of the other autolysins that were detected

INTRODUCTION

Bacterial autolysins that attack peptidoglycan (also termed murein hydrolases) are involved in cell wall synthesis and breakdown. The various kinds of bacterial autolysins have been reviewed by Höltje (1995). It is often difficult to demonstrate autolytic activity in cell-free extracts, possibly because a firm binding of the autolysins to cell wall components makes it difficult to obtain the free enzymes or because activation of the enzymes is needed. Treatment with sodium dodecyl sulfate (SDS) can release or activate autolysins and the denatured enzymes can be renatured with low ionic strength Tris-HCl buffer (Potvin et al., 1988). Thus the detection and separation of autolysins can be done by zymogram analysis, i.e., by incorporating a turbid substrate such as whole cells, cell walls, or purified peptidoglycan into a SDS-polyacrylamide gel before electrophoresis and then renaturing the enzymes after electrophoresis to allow them to produce bands of clearing (e.g., see Potvin et al. 1988, Laclerc and Asselin 1989, Bernadsky et al. 1994, Watt and Clarke 1994).

Autolysins of the members of the genus *Campylobacter* have not been studied to date. *Campylobacter upsaliensis* is a gram negative, microaerophilic, vibrioid bacterium found in the normal and diarrheic feces of dogs and cats (Burnens and Nicolet 1992, Sandstedt and Ursing 1991). In humans it can cause bacteremia (Babay et al. 1996, Carnahan et al. 1994, Chusid et al. 1990, Lastovica 1989), miscarriage (Gurgan and Diker 1994), abscesses (Gaudreau and Lamothe 1992), and diarrhea (Lindblom 1995, Megraud and Bonnet 1986). We previously characterized the conversion of the spiral form of *C. upsaliensis* cells from mid-log phase cultures to a nonculturable, coccoid form during incubation under an air atmosphere at 37°C (Santiwatanakul and Krieg 1998). Such a spiral-to-coccoid conversion occurs in various other helical bacteria such as *Campylobacter jejuni, Campylobacter coli*, and *Helicobacter pylori* (Tenover and Fennell 1992). It is possible that this morphological change might result from the activity of autolysins, particularly in view of the report by Amano and Shibata (1992) of the isolation of peptidoglycan from the spiral form of *C. coli* but not from the coccoid form.

In this study we report the detection and isolation of one of the autolysins of *C. upsaliensis* by zymogram analysis.

MATERIALS AND METHODS

Bacterial strain and culture conditions.

Campylobacter upsaliensis VPI strain CG-1 was obtained from C. Gebhart, Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, MN and was maintained aerobically at 37°C in semisolid Brucella agar (Difco) with weekly transfer. Cultures for autolysin study were obtained as follows. Two l of Brucella broth contained in a 4-l erlenmeyer flask were inoculated with 20 ml of a 48-h-old culture of CG-1 grown in Brucella broth at 37°C under an atmosphere of 6% O_2, 3% CO_2 and 91% N_2. The flask was incubated at 37°C under a similar gaseous atmosphere in a reciprocal shaking waterbath (40-45 oscillations per min). Cultures were collected at mid-log phase (22-24 h) and the spiral cells harvested by centrifugation. To obtain coccoid cells, however, some cultures were further incubated aerobically at 37°C for 4 days with continuous shaking on a rotary shaker at 100 rpm before harvesting the cells.

Preparation of autolysin-containing cell extracts.

The centrifuged cells were washed three times with 50 mM potassium phosphate buffer (PPB), pH 7.0, suspended in 1 ml of PPB and lysed by sonic oscillation. After centrifugation of the lysate at 130,000 × *g* for 1 h at 4°C, the soluble fraction of the lysate was collected and stored at -30°C. The sediment fraction was washed three times with PPB, suspended in PPB, and stored at -30°C. Protein concentrations in these preparations were estimated by the assay method of Bradford (1976).

Preparation of turbid substrates for autolysins.

Two different turbid substrates were used. The first was a 2% suspension of *Micrococcus luteus* (formerly *M. lysodeikticus)* cells (Worthington Biochemical Corp., Lakewood, NJ). The second consisted of murein sacculi (peptidoglycan) from *Escherichia*

coli ATCC 9637 prepared by a method adapted from de Jonge et al. (1992) and Glauner (1988). Peptidoglycan from *E. coli* was chosen because isolation from *C. upsaliensis* would have required very large volumes of culture because of the low cell densities produced by this organism; moreover, *E. coli*, which does grow to high cell densities, has a peptidoglycan that appears to be similar in composition to that of most other gram negative organisms (Labischinski and Maidhof 1994). Three 2-l cultures contained in 4-l erlenmeyer flasks were grown aerobically with shaking in a medium containing 1% trypticase, 1% glucose, 0.5% NaCl, and 0.25% K_2HPO_4 until they reached a turbidity of 1.5-1.6 at 600 nm. The cultures were rapidly chilled in ice water bath and centrifuged at 15,000 × *g* at 4°C for 10 min. The sediment was washed once with cold (4°C) distilled water and then suspended in 30 ml of cold distilled water. The suspension was added slowly to 500 ml of 4% SDS, boiled with stirring for 90 min, allowed to cool to room temperature, and centrifuged at 130,000 × *g* at 25°C for 60 min. The sediment was washed twice more with distilled water, suspended in 10 ml of distilled water, and heated for 2 min, centrifuged, and washed three times with distilled water. It was suspended in 8 ml of 100 mM Tris-HCl (pH 7.5) and 4 mg of α-amylase were added. After incubation at 37°C for 2 h, 0.4 mg of DNase I and 2 mg of RNase A were added. $MgSO_4$ was added to a final concentration of 20 mM and the mixture was incubated at 37°C for 2 h. Subsequently, 4 mg of trypsin was added. $CaCl_2$ was added to a final concentration of 10 mM and the mixture was incubated overnight. SDS was added to a final concentration of 1% and the mixture was boiled for 15 min, diluted with 20 ml of warm (60°C) distilled water, and centrifuged at 130,000 × *g* at 20°C for 10 min. The sediment was washed twice with distilled water, weighed, and suspended in distilled water to a final concentration of 0.2 g ml^{-1}. Sodium azide (0.02% final concentration) was added as a preservative.

Quantification of autolytic activity.

The assay was adapted from Flemming (1922). Portions (200 µl) of the soluble fraction of lysates were added to 3 ml of 25 mM Tris-HCl buffer pH 8.0 containing 100 µl of 2% *M. luteus* suspension and also to 200 µl of *E. coli* peptidoglycan suspension (0.2 g

ml^{-1}). The mixtures were incubated at 37°C for up to 24 h and any decreases in turbidity were measured at 540 nm. Various concentrations of commercial egg-white lysozyme were assayed in a similar manner as a control.

Detection of autolysin activity by non-denaturing PAGE.

In attempts to detect autolysins by gel electrophoresis without using SDS to denature the autolysins, the soluble and sediment fractions from *C. upsaliensis* lysates were electrophoresed in 8.5% polyacrylamide gels (lacking turbid substrate) at 110 V. Gels containing turbid substrate (0.2% *M. luteus* or 1% *E. coli* peptidoglycan) were then placed as an overlay on the polyacrylamide gel. The gels were kept in moist chamber for up to 24 h. Autolysin activity was seen as clear bands in the *M. luteus* substrate gel or as colorless bands in EC gels that were washed five times with deionized distilled water and two times with 0.01% KOH, and then stained by the method of Bernadsky (1994).

Zymogram analysis.

The zymogram analysis was adapted from the methods of Leclerc and Asselin (1989), Audy et al. (1989) and Potvin et al. (1988). The SDS-PAGE used the discontinuous buffer system of Laemmli (1970). Separating gels contained 12% acrylamide, 0.375 M Tris (pH 8.8), 0.1% SDS, and either 0.2 % (w/v) final concentration of *M. luteus* cells (termed ML gels) or 1% (w/v) final concentration of *E. coli* peptidoglycan (EC gels). Stacking gels consisted of 4% acrylamide, 0.125 M Tris (pH 6.8), and 0.2% SDS. The lysate or sediment per well contained 100 µg protein. Gels were electrophoresed in a Mighty Small™ vertical slab gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA) at 10 mA per gel for 3 h, followed by incubation for 16 h with gentle shaking at 37°C in 500 ml of 25 mM Tris-HCl buffer (pH 8) and 1% Triton X-100. With ML gels, autolytic activity appeared as a clear zone in a translucent background. EC gels were washed and stained as described above. Autolytic activity appeared as colorless zones against a blue background.

Autolysin isolation.

With ML gels, autolytic activity appeared as a single band at 34 kDa in a translucent background whereas on EC gels there were 14 autolytic bands, of which one corresponded to the 34 kDa band on the ML gels. This band, which occurred on both ML and EC gels, was chosen for autolysin isolation. If merely cut out of the gel for study, this 34 kDa band could have contained other proteins besides the autolysin, all having a similar molecular weight. In obtaining only the autolysin, it was reasoned that the autolysin would adhere to the walls of whole cells of *M. luteus,* unlike other contaminating proteins, and would be released later by SDS treatment. Consequently, the procedure outlined in Fig. 1 was used for isolation of the autolysin. Sonicated cells of *C. upsaliensis* were centrifuged at 130,000 × *g* for 1 h at 4°C and the soluble fraction was collected. One ml of 2% (w/v) whole cells of *M. luteus* was added to 100 µl of *C. upsaliensis* soluble fraction and also to a control (100 µl of PPB). The mixtures were incubated at 0°C (to prevent the autolysin from destroying the *M. luteus* cell walls) for 1 h and centrifuged at 130,000 × *g* at 4°C for 10 min to sediment the *M. luteus* cells. The surface of each sediment was washed twice with PPB and suspended in 100 µl of PPB. Thirty µl of sediment or supernate were added to a solution containing 4% SDS, 2% (v/v) 2-mercaptoethanol and 5% (w/v) sucrose (final concentrations). After boiling for one min, the mixtures were subjected to SDS-PAGE in gels lacking and containing *E. coli* peptidoglycan. (The SDS/boiling treatment liberates any autolysins, whether they are *C. upsaliensis* autolysins adsorbed onto the *M. luteus* cells or whether they belong to *M. luteus* itself). Gels without peptidoglycan were electrophoresed at 110 V and stained with Coomassie blue stain. Gels containing peptidoglycan were electrophoresed and stained as described by Bernadsky (1994). The autolysin band, indicated by a colorless zone at 34 kDa, was cut from the gel and its purity determined by isoelectric focusing (IEF), since SDS-PAGE gels stained with Coomassie blue would not be able to detect contaminating proteins because they would all have the same molecular weight.

For IEF, the 34 kDa band was added to 20 µl of PPB and minced with a motorized mixer. Twenty µl of sample buffer containing 9.5 M urea, 2% (v/v) Triton X-100, 5% (v/v) 2-mercaptoethanol, 4% (v/v) Ampholyte 5/7 (Bio-Rad), and 1% (v/v) Ampholyte

3/10 (Bio-Rad) was added to the minced band and allowed to stand at room temperature for 30 min before placing into the electrophoresis well. The isoelectric focusing vertical slab gel contained 9.2 M urea, 4% acrylamide, 3% Triton X-100 detergent, and 2% ampholytes. The upper buffer consisted of a cathode solution (20 mM NaOH) and the lower buffer consisted of an anode solution (20 mM H_3PO_4). The slab gel was isoelectrically focused with a 20-µl sample overlay containing 9.02 M urea, 2 % (v/v) Ampholyte 5/7 (Bio-Rad), 0.5% (v/v) Ampholyte 3/10 (Bio-Rad), and 0.0025% Bromphenol blue for 30 min at 250 V. Then the sample was loaded into the well and followed by 20 µl of sample overlay. The gel was electrophoresed at 10°C for 4 h at 500 V. The gel was soaked in 50% reagent-grade methanol for at least 1 h and stained with silver stain by method of Wray et al. (1981).

NH_2-terminal sequencing.

The purified autolysin was electrophoresed by SDS-PAGE with a gel containing *E. coli* peptidoglycan. Ten wells were used in order to obtain sufficient autolysin for analysis. After renaturing, staining and destaining, each 34 kDa colorless band was cut, minced, subjected to SDS-PAGE, again using 10 wells. The gel was transblotted onto a polyvinylidene difluoride membrane (Bio-Rad) using a Transphor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA) and stained with 0.025% Coomassie brilliant blue R 250. The transblotted bands were cut and the protein eluted. The protein was sequenced using a model 477A Sequenator (Applied Biosystems, Foster City, CA).

Production of polyclonal antibodies against the 34 kDa autolysin.

Antibodies specific to the 34 kDa autolysin were produced in 3.5-kg, female New Zealand white rabbit, which was first bled to obtain pre-immune serum. Three bands of purified autolysin containing approximately 100 µg of protein were individually emulsified in 500 µl of Ribi adjuvant (MPL+TDM+CWS, Ribi ImmunoChem Research, Inc., Hamilton, MT). Individual antigen preparations were injected into the rabbit on days 1, 42, and 84 via either the intramuscular or subcutaneous route. On day 94, the rabbit was euthanized by pentobarbital overdose followed by exsanguination via cardiac puncture.

The specificity of the antibodies obtained was tested by using Western blot (see below) of a two dimensional gel of the soluble fraction of the *C. upsaliensis* lysate and an immunoprecipitation following with zymogram (see below).

Western blotting.

Proteins were transferred from SDS-PAGE gels to nitrocellulose membranes by the method of Towbin et al. (1979). Proteins in the soluble fraction of the *C. upsaliensis* lysate and the purified 34 kDa autolysin were subjected to SDS-PAGE and then transferred by means of a Transphor™ apparatus to nitrocellulose membrane blotting paper (pore size 0.45 µm; Micron Separations Inc, Westborough, MA) for 1 h at 100 V. Nonspecific binding sites were blocked by treating the membrane with 5% bovine albumin in Tris-buffered saline (10 mM Tris [pH 7.2], 150 mM NaCl) overnight at 25°C. The membrane was washed twice with a washing buffer (0.02% [v/v] Tween 20 in Tris-buffer saline) and was incubated with diluted antiserum in 1% bovine albumin (in Tris-buffer saline) for 1 h at 25°C. After washing three times with washing buffer (10 min each wash), the membrane was incubated with diluted horseradish peroxidase conjugated goat anti-rabbit IgG for two h at 25°C. The membrane was washed three times with washing buffer and twice with deionized distilled water (10 min each wash). Chromogenic substrate solution consisting of 15 mg of 4-chloro-1-napthol (dissolved in 5 ml of methanol), 21 ml of Tris-saline buffer, and 6 µl of 30% H_2O_2 was added to the membrane. The color reaction was developed within 5-10 min. The membrane was washed with excess distilled water to stop further reaction.

Immunoinhibition test of antiserum against the 34 kDa autolysin

The supernate from a whole cell lysate of *Campylobacter upsaliensis* containing 200 µg protein was mixed with 25 µl of antiserum. A similar mixture was prepared with preimmune serum as a control. The mixtures were incubated at 4°C for 30 min and centrifuged at 130,000 × g for 10 min. The supernate and sediment were electrophoresed on ML gels along with supernate of whole cell lysate as described above.

RESULTS

Quantification and/or detection of autolytic activity under nondenaturing conditions

Despite repeated attempts, the soluble portion of *C. upsaliensis* lysates failed to cause any decrease in the turbidity of *M. luteus* suspensions or *E. coli* peptidoglycan suspensions. Commercial egg-white lysozyme did cause extensive loss of turbidity (data not shown). No autolysin activity was detected in non-denaturing PAGE gels overlaid with gels containing *M. luteus* cells or *E. coli* peptidoglycan as turbid substrates.

Detection of *C. upsaliensis* autolysins by SDS-PAGE followed by renaturation

Different patterns of autolytic bands occurred depending on the turbid substrate used in the renaturing gels. Gels containing whole cells of *M. luteus* (ML gels) showed only one autolytic clear band at 34 kDa (Fig. 2A) whereas gels containing *E. coli* peptidoglycan (EC gels) showed not only the 34 kDa band but also 13 other colorless bands ranging from 200 kDa to 12 kDa (Fig. 2B). Similar patterns were obtained with spiral cells and coccoid cells, and with the soluble fractions and the sediment fractions of lysates. The intensity of the bands (and presumably the autolytic activity), however, was less with the coccoid form compared with the spiral form, and with the sediment fractions compared with the soluble fractions.

Purity of the 34 kDa autolysin

As indicated in Materials and Methods, whole cells of *M. luteus* were used to selectively adsorb the 34 kDa autolysin from the soluble fraction of lysates. *M. luteus* had an 51 kDa autolysin of its own and was distinct from the *C. upsaliensis* 34 kDa autolysin (Fig. 4a, lane 1). Electrophoresis of the supernate and cell sediment of controls showed the absence of any detectable proteins having a molecular weight >31 kDa (Fig 3, lane 2 and 3). The binding of autolysin at 34 kDa band by whole cells of *M. luteus* left some residual autolysin in the supernate, as shown by SDS-PAGE of the supernate (In Fig. 3, compare lane 4 with lane 5). To make sure that the 34 kDa autolysin had been adsorbed,

zymograms of the *M. luteus* cell sediment in both the sample and the control were analyzed in EC renaturing gels. In the zymogram of the *M. luteus* cells that had been used to adsorb the 34 kDa autolysin, two autolytic colorless bands occurred at 51 and 34 kDa (Fig. 4A, lane 2). Only the 51 kDa band occurred in the control, indicating that this band was an *M. luteus* autolysin (Fig. 4A, lane 1). The 34 kDa band appeared to contain only one protein as indicated by the occurrence of a single protein band near the basic end of the isoelectric focusing slab gel (Fig. 4B). The 34 kDa autolysin was then used for rabbit immunization.

N-terminal sequence of 34 kDa autolysin

The first 15 residues of amino acids are given in Fig. 5 and are compared with the N-terminus of protein PEB4, which is an antigenic protein of *Campylobacter jejuni* described by Burucoa et al. (1995). There was 67% identity to the PEB4 N-terminal sequence when the data were analyzed by the BLAST search tool program (Altschul et al. 1990).

Specificity of the polyclonal rabbit antiserum to the 34 kDa autolysin

Western blotting of the antiserum to the 34 kDa autolysin indicated no cross-reactivity with other proteins in the soluble fraction of *C. upsaliensis* lysates (Fig. 6). Moreover, when the soluble fraction of a *C. upsaliensis* lysate was subjected to two-dimensional gel electrophoresis, silver staining revealed many proteins (Fig. 7A) whereas Western blotting showed only a single spot, a spot that corresponded to the 34 kDa autolysin (Fig. 7B).

The immunoinhibition test using the antiserum prepared against the 34 kDa autolysin indicated that the antiserum was able to precipitate this autolysin from the supernate of a cell lysate. After treatment of the supernate with the antiserum, the autolytic activity occurred in the sediment as shown by the development of a clear band following zymogram analysis (Fig. 8, lane 3). The supernate did not contain this activity (Fig. 8, lane 2). When preimmune serum was used instead of the antiserum, the opposite

result occured: the 34 kDa autolysin activity remained in the supernate (Fig. 8, lane 4) and did not occur in the sediment (Fig. 8, lane 5).

DISCUSSION

The failure to detect autolytic activity in suspensions of turbid substrate or by gel electrophoresis unless SDS treatment was used followed by renaturation could be attributable to a firm binding of the autolysin(s) to components of the cell wall, thereby precluding enzyme attachment to and lysis of the substrate particles. The SDS treatment would likely cause release from the bound state and thus allow electrophoretic separation. Alternatively, SDS treatment and subsequent renaturation might activate autolysins from a previously inactive state. For instance, denaturation and renaturation of muramidase-2 in *Enterococcus hirae* increases its activity (Shockman et al. 1996).

The occurrence of a single autolytic band in renaturing gels containing *M. luteus* cells as the turbid substrate, whereas 14 bands occurred in gels having purified *E. coli* peptidoglycan, may be due to the different compositions of the peptidoglycans of these organisms. Peptidoglycans of gram positive bacteria vary in their kind of diamino acid, the linkage (group A or group B) between the tetrapeptide chains, and the interpeptide bridges, if any, that may occur. The peptidoglycans of most gram negative bacteria appear to be similar to one another in composition in that they have diaminopimelic acid as the diamino acid, the Group A type of linkage, and no interpeptide bridges. Using gram positive cell walls as the turbid substrate, Leclerc and Asselin (1989) found that only one lytic band occurred when extracts of *Clostridium perfringens* were analyzed in the presence of homologous cell walls but 15 bands occurred when *M. luteus* cell walls were used. They suggested that the multiplicity of lytic bands may not necessarily have been a directly reflection of the number of cell wall hydrolases. This suggestion is supported by the work of Potvin et al. (1988), who showed that a unique cloned gene of *Bacillus* sp. could express several molecular forms of a cell hydrolase when analyzed in the same gel system. The multiple forms in fact represented cell wall hydrolases truncated at the C-terminal end of the intact protein. Band multiplicity may result from covalent

modification, such as glycosylation and nucleotidylation, or proteolytic processing (Dolinger et al. 1988). In the present study, however, if the 34 kDa band present in *E. coli* renaturing gels had possessed similarity to some part of the other lytic bands, the Western blotting with antiserum against 34 kDa should have shown more than one band. Since only a single specific band occurred, with a molecular weight of 34 kDa, it seems unlikely that the 34 kDa autolysin was derived from any of the other autolysins that were detected.

The significance of the similarity in N-terminal amino acid sequence to the amino acid sequence of positions 22 to 36 in the PEB4 protein of *Campylobacter jejuni* is not readily apparent, since the function of PEB4 is unknown. However, positions 1 to 22 of the PEB4 protein constitute a signal peptide that is characteristic of transported proteins, and a putative cleavage site for a signal peptidase occurred between positions 21 and 22. Therefore, it is likely that PEB4 protein can be transported across the cytoplasmic membrane of *C. jejuni*. This suggests that the *C. upsaliensis* autolysin may also occur in a precursor form with a signal peptide and that the isolated autolysin may have been processed by a signal peptidase.

In conclusion, this report shows how whole cells of one bacterium may be used to adsorb a specific autolysin from a mixture of autolysins and other proteins. The mode of action of the enzyme is not yet known, but the characterization of this and other autolysins in *C. upsaliensis* may eventually help to explain how cell wall growth occurs in this species or how morphological changes such as the transformation of the spiral form of *C. upsaliensis* to the nonculturable coccoid form take place. Several other autolysins also appear to exist in *C. upsaliensis* but they have not yet been characterized.

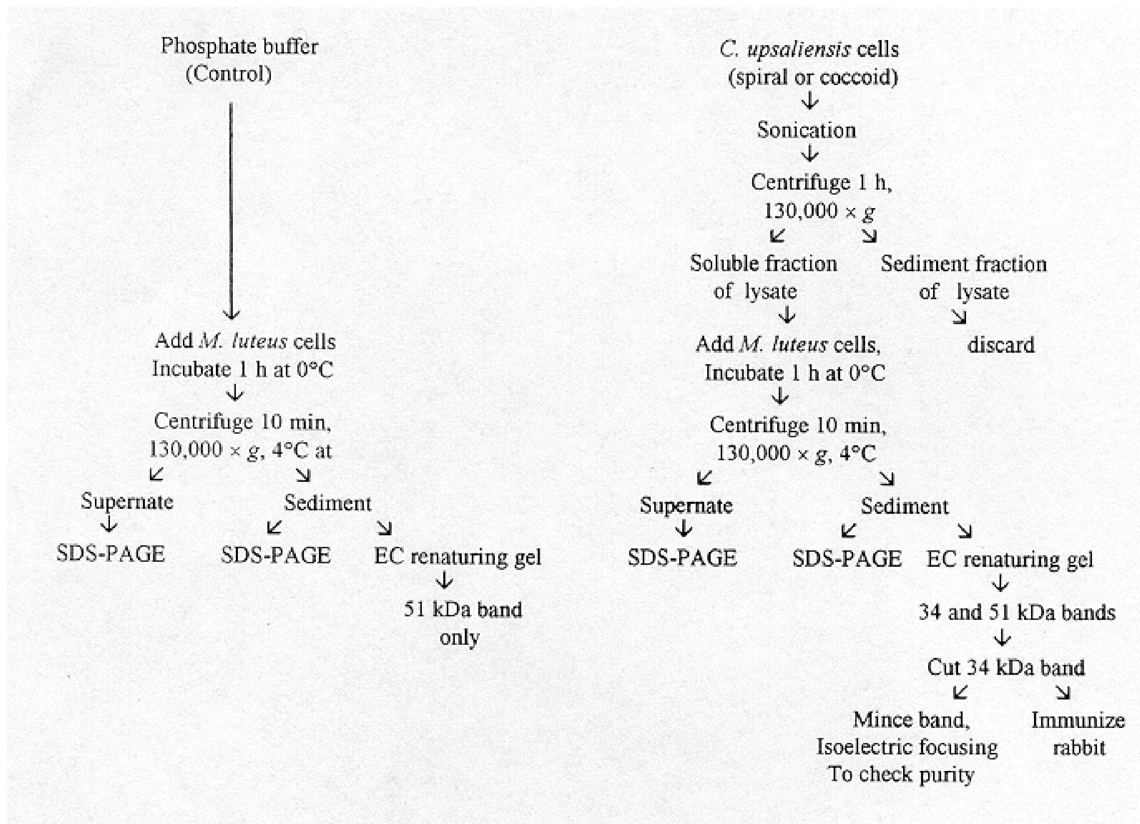

FIG. 1. Flow diagram for isolation of *C. upsaliensis* 34 kDa autolysin and for the control. The steps are described more fully in the text.

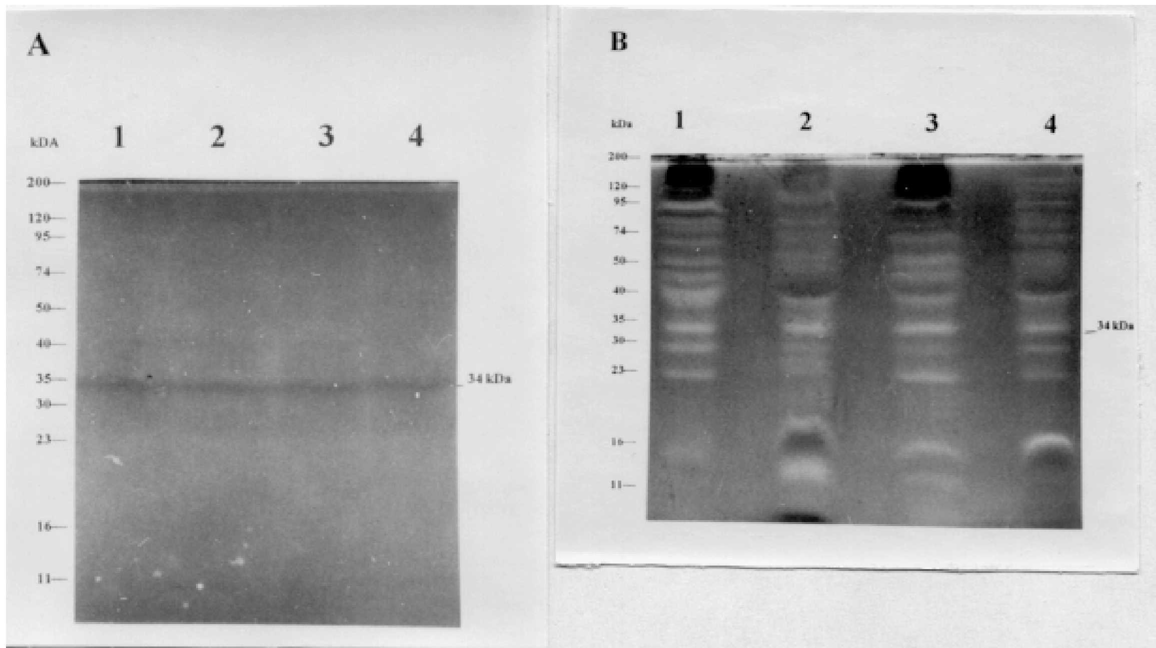

FIG. 2. Typical renatured gels showing *Campylobacter upsaliensis* autolysins. (A) Gel containing whole cells of *M. luteus*. A clear autolytic band (dark band in this photograph because the gel was placed against a black background) occurs at 34 kDa with the soluble fraction from spiral cells (lane 1) and coccoid cells (lane 3), and with the sediment from spiral cells (lane 2) and coccoid cells (lane 4). (B) Stained gel containing *E. coli* peptidoglycan, showing multiple autolytic colorless bands ranging from 200 kDa to 12 kDa with the soluble fraction from spiral cells (lane 1) and coccoid cells (lane 3) and with the sediment from spiral cells (lane 2) and coccoid cells (lane 4). Each lane contained 100 μg total protein.

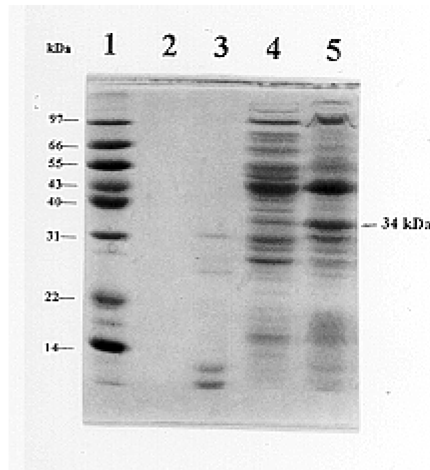

FIG. 3. Typical Coomassie blue-stained SDS polyacrylamide gel of the supernate from *Campylobacter upsaliensis* lysates adsorbed with whole cells of *Micrococcus luteus*, showing. Lane 1 is a standard molecular weight marker. Lanes 2 and 3 are controls: lane 2 shows lack of proteins in the supernate following removal of *M. luteus* from the control (buffer), and lane 3 shows the protein pattern upon electrophoresis of the intact whole cells of *M. luteus* that were removed from the control buffer. Lane 4 is the the supernate obtained after removing *M. luteus* from the *C. upsaliensis* soluble fraction, and lane 5 is the protein pattern upon electrophoresis of the intact whole cells of *M. luteus* that were removed from the soluble fraction. In lane 4 a decrease in the intensity of the band at 34 kDa can be seen compared to lane 5, indicating adsorption of 34 kDa protein(s) by the *M. luteus*.

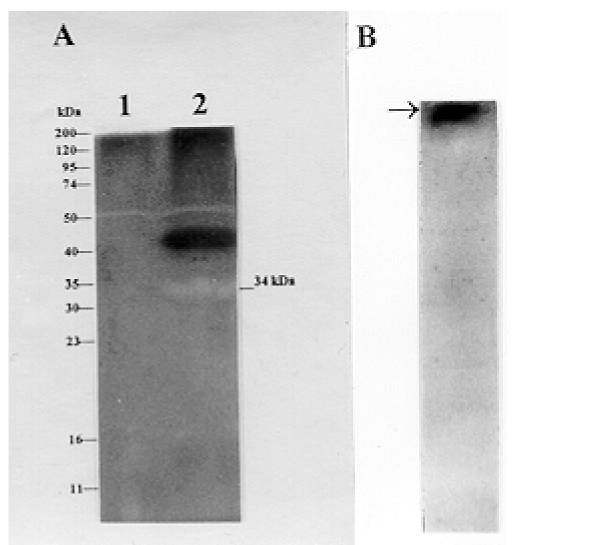

FIG. 4. (A) Renaturing gels containing *E. coli* peptidoglycan showed colorless autolytic bands. Lane 1, control *M. luteus* cells which have been centrifuged from buffer, showing an autolysin band at 51 kDa that is derived from *M. luteus* itself. Lane 2, *M. luteus* cells which have been centrifuged from the soluble fraction of lysates of *C. upsaliensis,* showing the 51 kDa *M. luteus* autolysin band and the 34 kDa *C. upsaliensis* autolysin band. (B) Minced 34 kDa *C. upsaliensis* autolysin band subjected to isoelectric focusing to test purity of the autolysin. Only a single protein band can be seen (arrow) after electrophoresis and silver staining.

```
                      1         5           10          15
34 kDa Autolysin      A T V A T V D G E K I G W T E
                      | |  | | | |•  |•   |      | |
PEB4                  A T V A T V N G K S I  S  D T E
                      22        26          31          36
```

FIG. 5. A comparison of the N-terminal sequences of the 34 kDa autolysin of *C. upsaliensis* and PEB4 protein of *C. jejuni*.

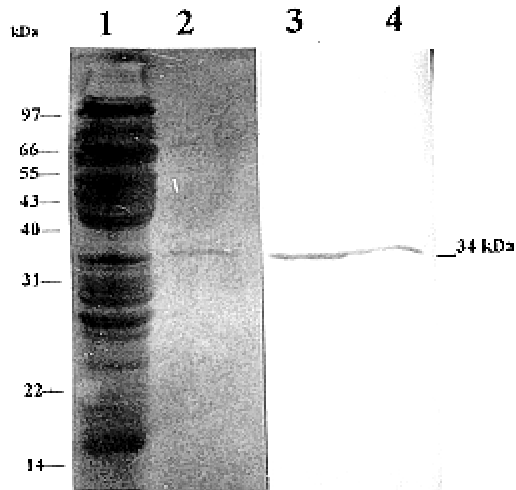

FIG. 6. Lane 1: Proteins from an SDS-PAGE of the soluble fraction (lane 1) transferred to a nitrocellulose membrane, stained with Coomassie Blue. Lane 2: Proteins from the minced band of the 34 kDa *C. upsaliensis* autolysin transferred to a nitocellulose membrane, stained with Coomassie Blue. Only a single band is evident. Lanes 3 and 4: same as lanes 1 and 2 except stained with antibody against the 34 kDa autolysin (immunoblots).

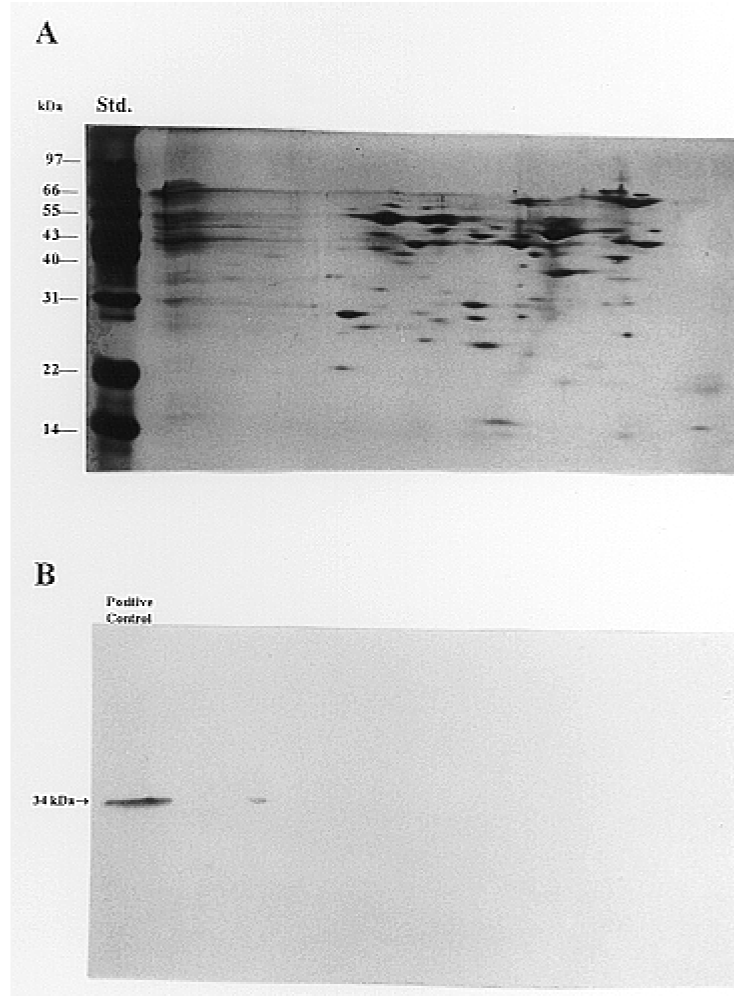

FIG. 7. (A) Silver-stained two dimensional gel of the soluble fraction of the *C. upsaliensis* lysate. The single lane at the left represents a mixture of molecular weight markers subjected to only one dimensional electrophoresis (SDS-PAGE). (B) Same as (A) except stained with antibody against the 34 kDa autolysin (immunoblot). The lane at the left represents a positive control consisting of the soluble fraction of the *C. upsaliensis* lysate, in which immunoblotting reveals only the 34 kDa band. In the two dimensional gel, only a single spot (S) is visible, corresponding in molecular weight to the 34 kDa band in the control.

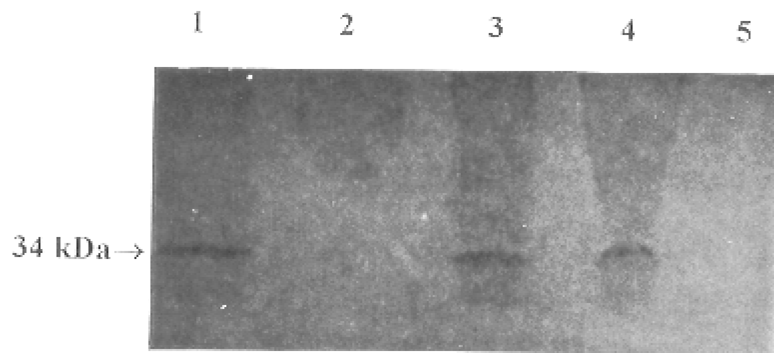

FIG. 8. Renaturing gel containing whole cells of *M. luteus* showing immunoinhibition test of antibodies against to 34 kDa autolysin activity of *C. upsaliensis*. Lane 1 is autolytic clear band of supernate of whole cell lysate. Lane 2 which is the supernate of immunoinhibition of antibodies against the 34 kDa autolysin did not show any autolytic clear band. Lane 3 which is the precipitate of antibodies against the 34 kDa autolysin demonstrated autolytic clear band at 34 kDa. Lane 4 which is the supernate after adding preimmune serum to the lysate demonstrated autolytic clear band at 34 kDa. Lane 5 which is the sediment after adding preimmune serum to the lysate did not show any autolytic activity.

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Summary and Conclusions

On the basis of experiments conducted in this study, the following conclusion were reached:

(i) The optimal temperature and time for converting the spiral form of *Campylobacter upsaliensis* to coccoid form under aerobic conditions are incubation at 37 °C for 4 days.

(ii) Chloramphenicol does not inhibit the conversion of the spiral to coccoid form.

(iii) Although the coccoid forms of *C. upsaliensis* shows fluorescence in acridine orange stain, they exhibit severe decreases in isocitrate dehydrogenase activity and in oxygen uptake with various substrates, and they show extensive degradation of ribosomal RNA. The coccoid form cannot be cultured after the second day of aerobic incubation.

(iv) It seems likely that the coccoid form of *C. upsaliensis* is a degenerative form rather than part of a life cycle, although it is difficult to prove this conclusively.

(v) The autolysins of *C. upsaliensis* cannot be detected by native gel electrophoresis.

(vi) Autolysins of *C. upsaliensis* can be detected by using the renaturing gel technique. A total of 14 autolytic colorless bands were demonstrated in stained gel, containing *E. coli* peptidoglycan as the

substrate (EL gel), whereas only one band was demonstrated in gels containing whole cells of *Micrococcus luteus* (ML gel).

(vii) A 34 kDa autolysin appeared in both EL and ML gels.

(viii) The 34 kDa autolysin was isolated by absorption of cell lysates with whole cells of *M. luteus*. The isolated 34 kDa autolysin appeared to have an isoelectric point in the basic range, as has been reported for other autolysins.

(ix) The N-terminal amino acid sequence analysis of the first 15 amino acids of 34 kDa autolysin showed 67% identity to a portion of antigenic protein PEB4 of *Campylobacter jejuni*. Since PEB4 is known to have a signal sequence for protein export, it is possible that the 34 kDa autolysin might be synthesized in a precursor form with a signal peptide, and that the latter is removed by a signal peptidase to give the mature protein.

(x) Antiserum prepared against the 34 kDa autolysin had high specificity and showed that the 34 kDa autolysin did not have antigenic similarity to any of the other autolysins of *C. upsaliensis*.

Future Areas of Investigation

The following areas deserve further investigation:

(i) The complete amino acid sequence of 34 kDa autolysin should be investigated and compared to the known amino acid sequences of other proteins.

(ii) The other autolysins of *Campylobacter upsaliensis* should be purified by using MonoQ anion exchange followed by R-0503 dye ligand chromatography as mentioned in the method of Watt and Clarke (1997).

(iii) The disulfide bonds in each purified autolysin should be investigated by the method of Watt and Clarke (1997).

(iv) The N-terminal amino acid sequence of each purified autolysin should be completed and compared to confirm whether some of the autolysins are related to others.

(v) Localization of each purified autolysin should be done by immuno-labeling technique.

(vi) Cloning of the gene for each purified autolysin could be initiated with the isolation of a DNA fragment with homology to a probe designed on the basis of the limited N-terminal sequence data.

Appendix A

Enzyme assays

Determination of enzymes activity in tricarboxylic acid cycle in the spiral form of *C. upsaliensis*

Cells from 1,500-ml of a 48-h-old culture of *C. upsaliensis* was harveseted by centrifugation and washed three times with 50 mM PBS, pH 7.4. The washed cells were suspended in 20 ml of 50 mM PBS, pH 7.4 and lysed by sonic oscillation. The lysate was centrifuged and the supernate was used for both enzyme assays and protein determination by the assay method of Bradford (1976). The lysate was kept in an ice bath during the assay or was frozen at -20°C for some enzyme assays. The assays were measured with a Bausch & Lomb Spectronic 2000 using a 1-cm quartz cuvette of 1 or 3 ml capacity: The tricaboxylic acid cycle enzymes assayed were citrate synthetase, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase, malate dehydrgenase, isocitrate lyase, and malate synthetase.

Either double or half volumes of cell extract were used to test linearity of acativity with protein concentration. Boiled (20 min) cell extracts were also tested to prove that activity was biological.

Citrate sythetase

This enzymes was assayed by method of Srere and et al. (1963). The 1-ml total volume of reaction mixture contained the following:

Tris-HCl buffer, pH 8.1, 400 mM	0.50 ml
5, 5'-bis thio (2-nitrobenzonic acid) (DTNB), 2 mM, freshly prepared in PBS, pH 7.0	0.10 ml
Acetyl-S-CoA, 2 mM, freshly prepared	0.10 ml
Potassium oxalate, 5 mM, freshly prepared	0.20 ml
Cell extract	0.10 ml

All reagents except oxalate and acetyl-S-CoA were mixed together and measured as a blank at an absorbance of 412 nm. Then the acetyl-S-CoA was added and the increase in absorbance (dued to acetyl-S-CoA deacylase activity) was recorded. After 2 min the

oxalate was added and the rate of increase in absorbance was recorded**.** The end product, thionitrobenzoate, has a molar extinction coefficient of 13,600 at 412 nm.

Aconitase

This enzyme was assayed by method of Racker (1950). The 1-ml reaction mixture contained the following:

Tris-HCl buffer, pH 7.5, 500 mM	0.20 ml
DL-sodium isocitrate, 200 mM	0.10 ml
Cell extract	0.10 ml
Distilled water	0.60 ml

All reagents except isocitrate were mixed together as a blank at absorbance of 240 nm. The reaction was initiated by addition of isocitrate and monitored as the rate of increase in absorbance. A change of 0.001 absorbance units was equivalent to 2.8×10^{-4} µmoles of cis-aconitate per ml.

Isocitrate dehydrogenase

This enzyme assaysed was modified from the method of Daron (1966) by reducing the concentration of $MnCl_2.4H_2O$ from 40 mM to 1 mM and increasing the concentration of DL-sodium isocitrate from 10 mM to 50 mM. NADP solution was prepared in distilled water and stored at -20°C for no more than one month. The 1-ml reaction mixture contained the following:

Tris-HCl buffer, pH 7.5, 200 mM	0.10 ml
$MnCl_2.4H_2O$, 1 mM	0.05 ml
NADP, 10 mM	0.05 ml
DL-sodium isocitrate, 50 mM	0.05 ml
Cell extract	0.025 ml
Distilled water	0.725 ml

All reagents except NADP were mixed together as a blank at absorbance of 338 nm. The reaction was initiated by addition of NADP with increasing of absorbance.

α-Ketoglutarate dehydrogenase

The method of Amarasingham and Davis (1965) was used for this assay. This enzyme must be assayed as soon as possible after preparation of the cell extract because of its instability. The NAD solution was prepared in distilled water and stored at -20 °C. The 3-ml reaction mixture contained the following:

Tris-HCl buffer, pH 8.5, 500 mM	1.00 ml
NAD, 60 mM	0.20 ml
CoASH, 2.6 mM, freshly prepared	0.10 ml
L-cysteine monohydrate, 78 mM, freshly prepared	0.10 ml
Thiamine pyrophosphate (TPP), 10 mM freshly prepared	0.10 ml
$MgCl_2$, 10 mM	0.10 ml
α-ketoglutarate, 250 mM	0.10 ml
Cell extract	0.10 ml
Distilled water	1.20 ml

The mixture without NAD was measured as a blank at absorbance of 338 nM. The increased rate of absorbance after adding NAD was recorded.

Succinate dehydrogenase

This assay was adopted from the method of Veeger and et al. (1969). This enzyme must be assayed as soon as possible after cell disruption. The 3-ml reaction mixture contained the following:

Potassium phosphate buffer, pH 7.6, 300 mM	1.00 ml
K_3EDTA, 30 mM	0.10 ml
KCN, 30 mM	0.10 ml
Potassium succinate, 400 mM	0.30 ml
Bovine serum albumin (BSA), 3.0% (W/V)	0.10 ml
$K_3Fe(CN)_6$, 75 mM, in foil covered tube	0.20 ml
Triton X-100, 3.0% (v/v)	0.10 ml
Cell extract	0.10 ml
Distilled water	0.90 ml

A blank was prepared without $K_3Fe(CN)_6$ and potassium succinate. $K_3Fe(CN)_6$ was added and the reaction mixture was incubated 3 min. After adding the potassium succinate, reduction of $K_3Fe(CN)_6$ was measured as the rate of decrease in absorbance at 455 nm ($\varepsilon = 150\ M^{-1}cm^{-1}$).

Fumarase

This assay was modified from method of Kanarek and Hill (1964) by using (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) instead of sodium phosphate buffer. The 3 ml total composition of reaction mixture was as following:

BES buffer, pH 7.3, 150 mM	1.00 ml
L(-)malic acid, pH 7.3, 150 mM	1.00 ml
Cell extract	0.05 ml
Distilled water	0.95 ml

A blank was prepared without malic acid. The rate of increase at 250 nm after adding of malic acid was monitored. A change of 0.003 absorbance units was equivalent to 8.46×10^{-4} µmoles of fumarate formed per ml.

Malate dehydrogenase

This enzyme was assayed by method of Ochoa (1955). The NADH was prepared in 0.001 N NaOH. The 1 ml total composition of reaction mixture was as following:

Potassium phosphate buffer, pH 7.4, 250 mM	0.50 ml
Potassium oxalate, 7.5 mM, freshly prepared	0.10 ml
NADH, 1.5 mM	0.20 ml
Cell extract	0.10 ml
Distilled water	0.20 ml

A blank was prepared without NADH and potassium oxalate. NADH was then added and endogenous activity was recorded as a decrease in absorbance at 338 nm. After 2 min potassium oxalate was added and the rate of decrease in absorbance was recorded.

Isocitrate lyase

This enzyme was assayed by method of Kornberg and Dixon (1959). The 3-ml reaction mixture contained the following:

Tris-HCl buffer, pH 7.5, 500 mM	0.20 ml
Phenylhydrazine.HCl, 100 mM, 100 mM, freshly prepared	0.20 ml
Cysteine.HCl, 1mM freshly prepared	0.10 ml
$MgCl_2$, 50 mM	0.20 ml
Potassium isocitrate, 20 mM	0.10 ml
Cell extract	0.10 ml
Distilled water	2.10 ml

A blank was prepared without potassium isocitrate. The potassium isocitrate was added and the rate of increase in absorbance at 324 nm was recorded. A change in absorbance of 11.55 absorbance units per min was equivalent to formation of 1 µmole of the complex per ml.

Malate synthetase

The enzyme was assayed by method of Eggerer and Klette (1967). The 3-ml reaction mixture contained the following:

Pyrophosphate buffer, pH 8.0, 20 mM	1.00 ml
$MgCl_2$, 50 mM	0.10 ml
K_3EDTA, 10 mM	0.10 ml
Acetyl-S-CoA, 2 mM, freshly prepared	0.10 ml
Sodium glyoxylate, 10 mM	0.10 ml
Cell extract	0.10 ml
Distilled water	1.50 ml

A blank was prepared without acetyl-S-CoA and sodium glyoxylate. Acetyl-S-CoA was added and any decrease in absorbance at 232 nm, due to acetyl-S-CoA deacylase, was recorded. Glyoxylate was added and the decrease of absorbance wwas measured. A change of 0.1 absorbance unit per min represents 0.031 µmoles of acetyl-S-CoA cleaved per ml.

Appendix B

RNA isolation and electrophoresis

RNA isolation and electrophoresis

Total RNA was isolated by the guanidine isothiocyanate isolation procedure described by Johnson (1994). The major problem in working with RNA was maintaining RNase-free conditions. One should first have an RNase-free preparation of RNA and then avoided introducing RNase during any of experiment steps. Therefore, using gloves and not touching the inside of RNase-free plasticware and glassware is nescessary to avoid contamination by RNase.

Material and Solutions for RNA isolation

Glassware: All glassware should be baked before using, to remove nucleases.

Plasticware: Nucleases from plasticware were removed by soaking in 0.02% diethyl pyrocarbonate solution overnight. The solution was decanted and the plasticware was autoclaved at 121 °C for 45 min.

Treated water: Diethyl pyrocarbonate was added to distilled water to a final concentration of 0.05 %. The water was kept overnight and then autoclaved at 121 °C for 45 min. This treated water was used for making up solutions and buffers.

TE buffer pH 8.0: The buffer contained 10 mM Tris-HCl pH 8.0 and 1 mM Na_2-EDTA.

TE buffer pH 7.5: The buffer contained 10 mM Tris-HCl pH 7.5 and 1 mM Na_2-EDTA.

Suspending buffer: This buffer contained 0.35 M sucrose (59.9g/500 ml) in TE buffer and was stored in refrigerator. Before using, the buffer was boiled in a water bath for 2 min to drive off most of the oxygen. When the

buffer was cool, 2-mercaptoethanol was added to a final concentration of 5 mM (37 µl/100 ml).

SDS-TE buffer: TE buffer pH 7.5 containing 0.5 % sodium lauryl sulfate.

Lysozyme solution: The chicken egg white lysozyme (Sigma) was used at a concentration of 5 mg/ml in TE buffer, pH 8.0.

Lysing solution: This solution contained 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, and 0.1 M mercaptoethanol.

RNase inhibitor: Recombinant ribonuclease inhibitor (ICN) was used.

Other solutions: 2 M sodium acetate (pH 4.0)
3 M sodium acetate (pH 6.0).
Water-aturated phenol,pH 4.3 ± 0.2 (Fisher)
Chloroform containing no isoamyl alcohol.
7.5 M ammonium acetate (Fisher).

Method of RNA isolation

C. upsaliensis cells from a 200-ml culture grown in Brucella broth were harvested by centrifugation at 7,500 × *g* for 10 min. The superantant was decanted and remaining medium was removed with a pasteur pipette. The cells were suspended in 2.5 ml of suspending buffer and placed in 125-ml ground-glass-stoppered flask., followed by addition of 2 µl of RNase inhibitor and 50 µl of lysozyme. The cells were digested at 37° C for 5 min. The digested cells were lysed by adding 10 ml of lysing buffer and mixing for 2 min. One ml of 2 M sodium acetate (pH 4.0), 10 ml of phenol and 2 ml of chloroform were added and mixed for 2 min. The mixture was transferred to a screw-cap centrifuge tube and centrifuged at 20,000 × *g* for 10 min. The upper aqueous layer was transferred to

a clean screw-cap centrifuge tube and 10 ml of isopropanol was added to precipitate the RNA. The RNA was pelleted at 20,000 × *g* for 10 min and the superante was discarded. The RNA pellet was dissolved in 10 ml of TE buffer pH 7.5 and 5 ml of 7.5 M ammonium acetate solution was added. The mixture was agitated vigorously and then placed on ice for 10 min. The mixture was centrifuged at 13,000 × *g* for 25 min and the pellet was retained and dissolved 2 ml of SDS-TE buffer. After addition of 2 ml of chloroform with agitation, the mixture was centrifuged at 20,000 × *g* for 10 min. The upper aqueous portion was transferred to a clean screw-cap centrifuge tube, 200 µl of 3 M sodium acetate (pH 6.0) was added with agitation. The RNA was precipitated with 2 ml of isopropanol and then centrifuged at 20,000 × *g* for 10 min. The supernate was discarded and the pellet was washed with 70 % ethanol. The pellet was completely dried and dissolved in 100 µl of treated water. The isolated RNA was diluted 1:50 by adding 20 µl of RNA to 980 ml of TE buffer (pH 7.5). The diluted RNA was measured at 260 nm and the concentration was calculated by the following formula:

$$\underset{\text{(mg/ml)}}{\text{RNA concentration}} = (OD_{260} / 23) \times \text{dilution factor}$$

Material and Soltuion for RNA electrophoresis

10 × MOPS buffer: This buffer contained 0.4 M MOPS pH 7.0, 0.2 M sodium acetate and 0.01 M Na_2-EDTA.

Ethidium bromide solution: Ten mg of ethidium bromide was dissolved in 1 ml of treated water and stored at 4 °C.

1% agarose gel: The 0.6 g of high melting agarose DNA grade which was Dnase-free and RNase-free was dissolved in 6 ml of 10×

MOPS and 54 ml of treated water. The mixture was boiled until the agar dissolved, cooled to 50 °C, and then 2 µl of ethidium bromide solution was added. The agar was cast on the gel caster and allowed to set for at least 20 min at room temperature.

RNA loading buffer: The loading buffer contained 50% glycerol, 80% (v/v) formamide, 1 mM Na_2-EDTA pH 8.0, 0.1% (w/v) bromphenol blue, and 0.1% (w/v) xylenecyanole FF.

Running buffer: The running buffer contained 15 % formaldehyde in 1 × MOPS buffer.

Method of RNA electrophoresis

The 1 % agarose gel was pre-electrophoresed in running buffer at 60 V for 15 min. The 10 µg of RNA was added with loading buffer (1 µl loading sample / 5 µl of sample) and incubated at 50 °C for 12 min. The sample was loading in to the gel and electrophoresed at 80 V for 1-1.5 h. The gel was photographed under the UV lamp.

Appendix C

Transmission electron microscope and Immunogold labeling for localization of 34 kDa autolysin

Transmission electron microscopy.

Conventional embedding was based on the method of Li et al. (1996). Mid-log phase cultures of spiral cells or 4-day-old cultures of the coccoid cells of *Campylobacter upsaliensis* were harvested at 7,000 × *g* and washed once with 20 mM sodium phosphate buffered saline (PBS), pH 7.2. The cells were enrobed in 2% (w/v) agarose on an grease-free glass-slide. A second method of embedding was also used in which the cells were fixed prior to being enrobed in agarose. In either method, the agarose was cut into 1 mm^2 cubes and fixed for 1 h at 4°C in a solution containing 0.1% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in 100 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 6.8. The specimens were washed twice with HEPES buffer and once with distilled water and incubated in 2% (w/v) uranyl acetate solution for 45 min at 4°C. They were dehydrated in 75 and 90% (v/v) ethanol (15 min each) at 4°C and in 100% ethanol (two 15-min periods) at 4°C. The dehydrated specimens were soaked for 4 h in 50% LR White (London Resin Company, Ltd., Berkshire, England) and overnight in 100% LR White. They were placed in fresh 100% LR White for 2 h at 60°C to polymerize the resin. Sectioning was done with a MT6000-XZ ultramicrotome (RMC. INC., Tucson, Arizona) and the sections were collected on nickel grids (mesh size, 400 µm) coated with carbon Formvar.

Immunogold labeling

For immunogold labeling, the sample grids were treated with 0.5% (w/v) glycine and then with 1% (w/v) bovine albumin (dissolved in PBS) to block nonspecific binding sites (30 min each treatment). The grids were incubated in suitably diluted antiserum for 1 h, washed five times with distilled water, and incubated with diluted protein A-gold (10-nm diameter; Sigma) for 30 min, and washed five times with distilled water. The grids were stained with 1% (w/v) uranyl acetate solution for 10 min and examined with a Zeiss EM10CR transmission electron microscope at 80 kV with the anti-contamination device in place.

Result

Localization of the 34 kDa autolysin by transmission electron microscopy

Thin sections of spiral cells (Fig. 1) and coccoid cells (Fig. 2) treated with Protein A-gold alone and with preimmune serum followed by Protein A-gold failed to show any gold particles (Fig. 1 B and C, Fig. 2 B and C). Gold particles were present in preparations treated with antiserum followed by Protein A-gold (Fig. 1A, Fig2A, respectively), but there was no definite association with any cellular compartments such as the outer membrane, cytoplasmic membrane, and periplasmic membrane. Similar results were obtained with both of the fixation methods employed.

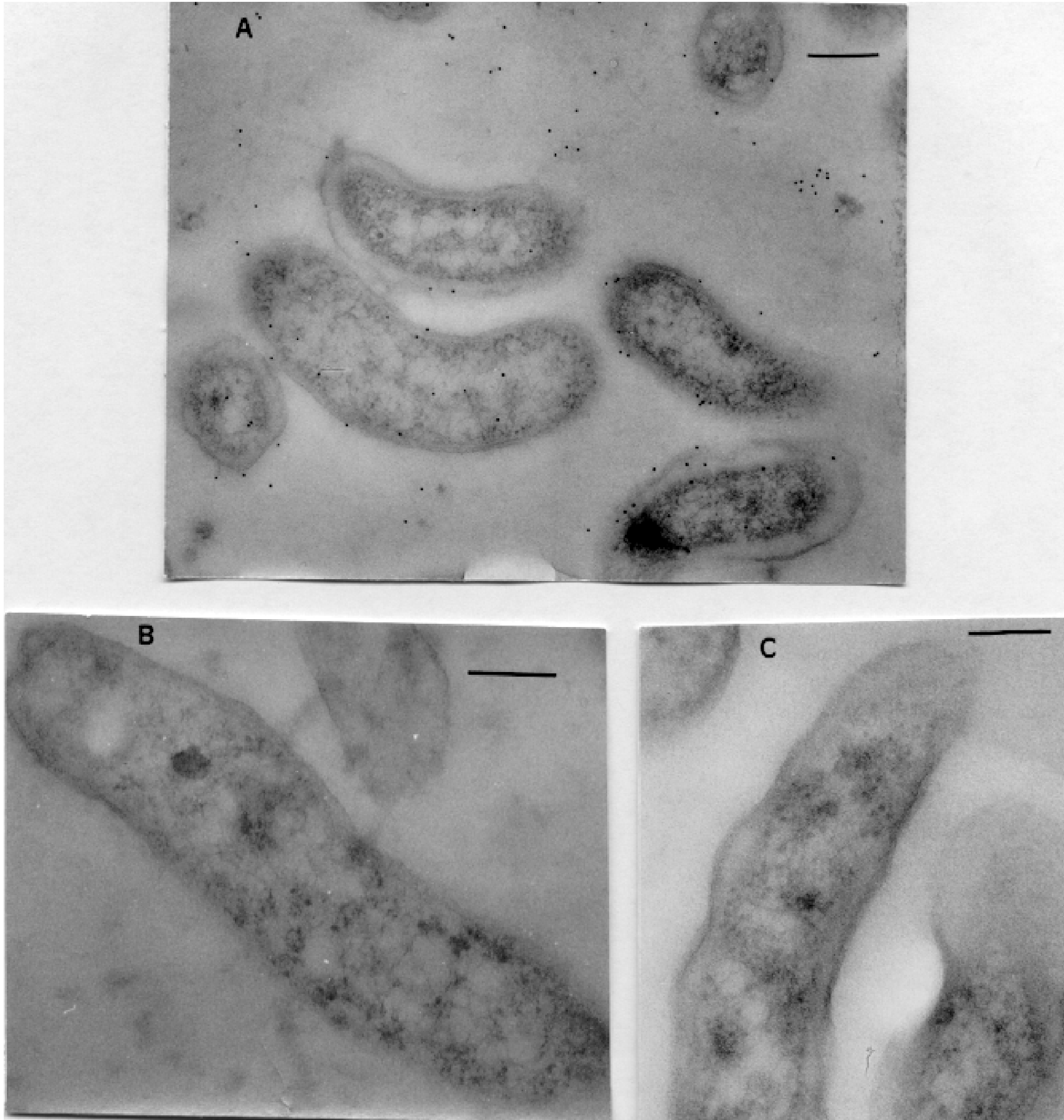

FIG. 1. Electron micrograph of thin sections of spiral cells of *C. upsaliensis*. (A), thin sections treated with antibodies against the 34 kDa autolysin and then Protein A-gold. No association of the gold particles with particular cell structures is evident. (B) and (C), control thinsections treated only with Protein A-gold or with preimmune serum and then Protein A-gold, respectively. Bar, 200 nm.

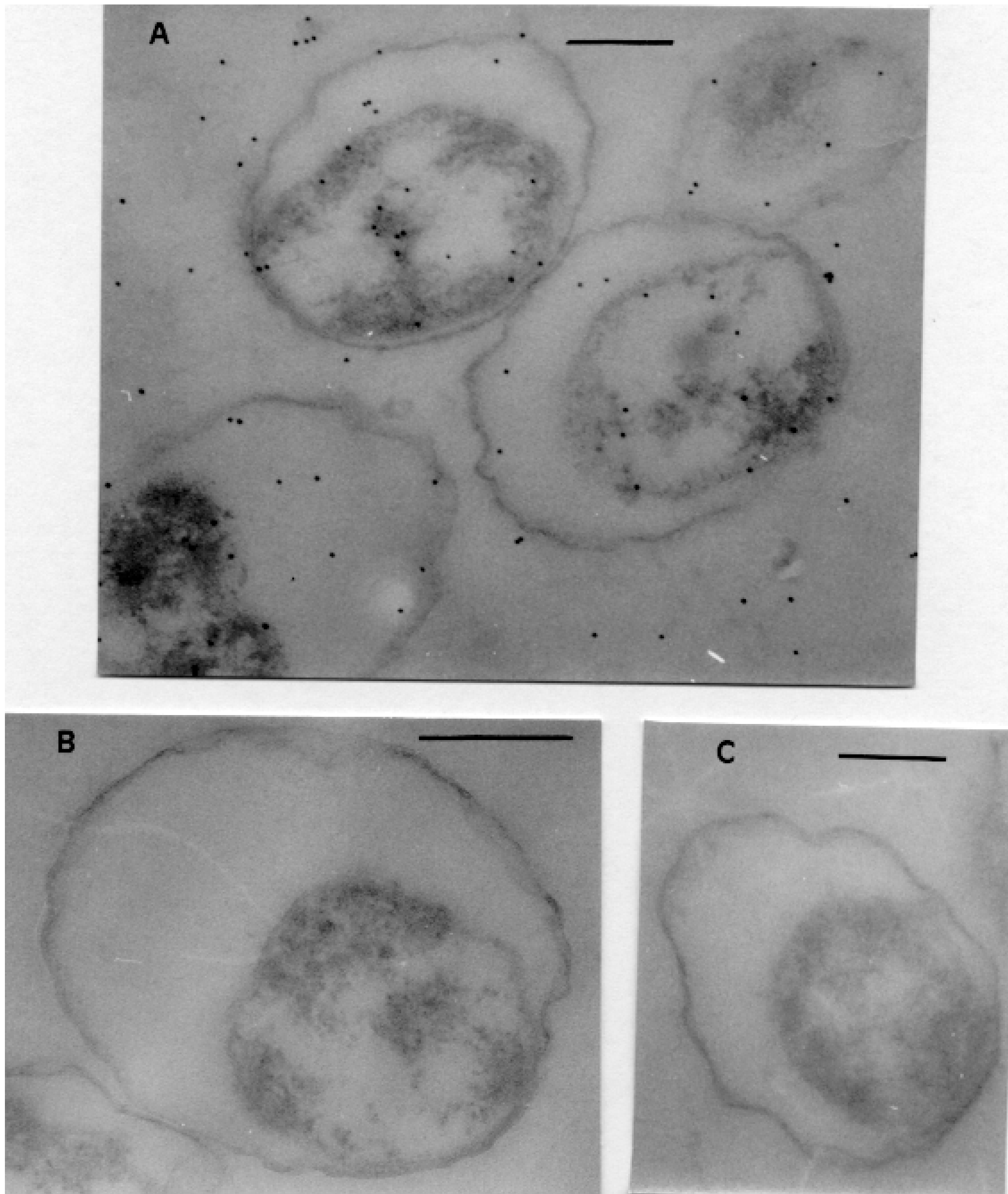

FIG. 2. Electronmicrograph of thin sections of coccoid cells of *C. upsaliensis*. (A), thinsections treated with antibodies against the 34 kDa autolysin and then Protein A-gold. No association of the gold particles with particular cell structures is evident. (B) and (C), control thinsections treated only with Protein A-gold or with preimmune serum and then Protein A-gold, respectively. Bar, 200 nm

Discussion

Based on immunogold electron microscopy, the 34 kDa autolysin of *C. upsaliensis* appeared to occur in the cytoplasm, cytoplasmic membrane, periplasm and outer membrane, which is in accord with the finding of activity in both the soluble fraction of lysates and in the sediment fraction. The lack of a specific location is in contrast to the results of Li et al. (1996), who showed by immunogold electron microscopy that a 26 kDa autolysin in *Pseudomonas aeruginosa* occurred mainly in the periplasm, although some was present in the cytoplasm as well. Also, Shockman and Höltje (1994) reported that peptidoglycan hydrolases are transported across the cytoplasmic membrane and become attached to the outer surface of the cytoplasmic membrane or bound directly to the peptidoglycan. However, Pooley and Shockman (1969) found that up to 85% of an autolysin of *Streptococcus faecalis* was in a latent form. They suggested that autolysin was synthesized and mainly located in the cytoplasm as an inactive precursor (proenzyme) which was then transported to sites on the cell wall associated with the wall biosynthesis, where it became active. The 34 kDa autolysin may be a similar enzyme, which could explain the failure to show the enzyme activity in native gels. The binding of antibodies to both the inactive and active form could explain the apparent lack of a specific location in the cells.

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