PLASMIDS IN *CLOSTRIDIUM BOTULINUM* TYPE A AND *CLOSTRIDIUM SPOROGENES*

by

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INTRODUCTION

Clostridium botulinum type A and *Clostridium sporogenes* are almost identical anaerobic sporeforming microorganisms. Their spores are highly resistant to drying and heating and therefore can cause problems in food processing. *C. botulinum* type A produces a neurotoxin which is the only general characteristic known to distinguish it from *C. sporogenes* which is nontoxic.

Botulism, a neuroparalytic disease, is usually the result of the ingestion of the toxin produced by *C. botulinum* in improperly processed food. Prevention of foodborne botulism therefore requires special treatment of many canned and vacuum packaged foods.

C. sporogenes, being proteolytic, also has great potential for food spoilage, if its spores are not killed in food processes which necessitate such a treatment. This food related problem stimulates the need to further study the unique toxin produced by *C. botulinum* and the relation of this bacterium and its toxin to *C. sporogenes*.

Many attempts to differentiate *C. botulinum* type A and *C. sporogenes*, have proven these species to be indistinguishable, without toxin testing. Biochemical, serological, nutritional, and genetic studies have provided data to substantiate the theory that *C. sporogenes* is a nontoxic variant of *C. botulinum* type A.

Toxin production has been described in *C. botulinum* types C and D as being bacteriophage-mediated. Bacteriophage isolated from other types of *C. botulinum* (A,B,E, and F), have not been associated with toxin production. Extrachromosomal DNA has been detected in toxic and

nontoxic type E strains and in one type A strain of C. botulinum (Scott and Duncan, 1978).

The objectives of this study were to develop a rapid screening procedure for plasmids in C. botulinum type A and C. sporogenes, determine the plasmid profiles of these bacteria, identify any relationship of plasmids to toxin production in C. botulinum type A, and evaluate whether C. botulinum type A and C. sporogenes can be differentiated based on plasmid profiles.

REVIEW OF LITERATURE

A. *Clostridium botulinum* and *Clostridium sporogenes*

Clostridium botulinum and *Clostridium sporogenes* are gram-positive, anaerobic, sporeforming, rod-shaped microorganisms that are commonly associated with the soil (Smith, 1975). *C. botulinum* has been known to be the causative agent in cases of food-borne human botulism since the late 1880's and has long been of concern to food processors. Specific heating processes and additives are now used in many canned and vacuum packaged foods to prevent botulism. On the basis of the serological and physiological characteristics of the organisms, *C. botulinum* is divided into three groups (Smith and Holdeman, 1968) and according to the serological specificity of the toxin produced, *C. botulinum* is divided into seven types (A-G). Group I includes the proteolytic types A, B, and F. Group II includes the nonproteolytic types B, E, and F. Group III is made up of toxin types C and D.

C. sporogenes closely resembles *C. botulinum* type A in all characteristics except for toxin production and therefore has been theorized as being a nontoxic variant of *C. botulinum* type A (Hadley, 1927; Gunnison and Meyer, 1929; Kindler et al., 1956; Wu et al., 1972).

Reddish (1921) noted that when *C. botulinum* and *C. sporogenes* were present in the same material, they were so closely associated that it was impossible to separate them. Hadley (1927) offered bacterial convergence as an explanation for this phenomenon, but it has never been shown that toxigenicity is transferable between *C. botulinum* type

A and C. sporogenes.

Serological studies have repeatedly experienced cross-reactions with C. botulinum and C. sporogenes. Cross-reactions with Clostridium parabotulinum (the old name for proteolytic strains of C. botulinum) are only eliminated after preabsorption of the C. sporogenes somatic antisera, but not all strains of C. sporogenes react uniformly (McCoy and McClung, 1938). Partial cross-agglutination of the somatic antisera of the proteolytic C. botulinum types and C. sporogenes, Clostridium tetani, and Clostridium histolyticum, has also been reported (Solomon et al., 1971). In studies to serologically group the proteolytic clostridia, some strains of C. parabotulinum and C. sporogenes have been shown to share heat labile (Mandia, 1951) and heat stable antigenic factors (Mandia, 1955). Nutritional studies with C. botulinum, C. parabotulinum, and C. sporogenes, concluded that the similarity in cultural and biochemical properties makes C. sporogenes indistinguishable from nontoxigenic strains of C. parabotulinum types A and B (Kindler et al., 1956). Also, it is not possible to differentiate between C. botulinum, C. sporogenes, and Clostridium perfringens based on extracellular proteases (Tjaberg and Fossum, 1973). C. botulinum type A and C. sporogenes showed the same amino acid utilization patterns in a study reported by Mead (1971) and similar end products from the metabolism of aromatic amino acids (Elsden et al., 1976).

Fluorescent antibody studies with C. botulinum and C. sporogenes have produced conflicting results. Walker and Batty (1964) were able to distinguish between the two bacteria, but noted that cross-reactions previously reported by Bulatova and Kabanova (1960) would make the

testing of more strains necessary before absolute certainty of differentiation could be established. Cross-reactions could have been eliminated by the selective absorption of unwanted C. sporogenes somatic antigens (Boothroyd and Georgala, 1964; Georgala and Boothroyd, 1967), but immunofluorescence techniques have not proven useful for the differentiation of these toxic and nontoxic organisms.

DNA and RNA homology studies have concluded that C. botulinum type A and C. sporogenes are genetically related. Lee and Riemann (1972), reported 50-100% homology between C. botulinum 62A and six strains of C. sporogenes. The thermal stability of the DNA hybrids studied by Lee and Riemann (1972) was reported by Wu et al. (1972) and they confirmed the earlier homology work. The DNA hybrids of the C. botulinum and C. sporogenes strains examined by Wu et al. (1972) were homologous and it was concluded that some of the toxic and nontoxic strains were closely related. The studies indicated that strains which are closely related serologically and biochemically are also closely related genetically and vice versa. Nakamura et al. (1977), using DNA reassociation between sixty-two C. sporogenes isolates from canned foods and C. botulinum type A190, concurred with all of the previously reported DNA homology studies. (Strain types 62A and A190 are the C. botulinum type A strains frequently used in food research in the United States and Japan respectively). Johnson and Francis (1975), reported that proteolytic strains of C. botulinum and C. sporogenes had ribosomal RNA homologies of 100%.

Kiritani et al. (1973) did a numerical taxonomy study with C. botulinum and C. sporogenes, which encompassed a wide range of bacterio-

logical characteristics, including enzyme activity, antibiotic sensitivity, cell morphology, acid production by fermentation, and other biological tests. Strains grouped within one phenon (groups with similar characteristics) were separated from those of the other phenons at a level of less than 77% S-value. The results of this work placed the proteolytic strains of C. botulinum and C. sporogenes in the same phenon with an S-value of 86%. This study confirmed that these two organisms have numerous common features.

It is now commonly accepted that proteolytic strains of C. botulinum and C. sporogenes are morphologically, serologically, and culturally indistinguishable except for neurotoxin production (Smith, 1975).

There are two reports of C. botulinum type A and C. sporogenes differentiation without testing for toxicity. Gas-liquid chromatography was used to compare trimethylsilyl derivatives prepared from whole-cell hydrolysates of either bacterium (Farshy and Moss, 1970). Differences were based on the ratio of two peaks, corresponding to arabinose and glucose, but only a few species could be compared at a time because of the complexity of the chromatograms. More recently, proteolytic C. botulinum types A, B, and F were differentiated from C. sporogenes when soluble cellular protein patterns were compared in polyacrylamide gels (Cato et al., 1982). The banding in the gels gives distinctive phenotypic evidence of the similarity within the species. The ability to differentiate the organisms by gel patterns was successful in that two bands, one at 4 mm migration distance and a dark band at 62 mm, were present in cultures of C. sporogenes and absent in the toxic strains of C. botulinum. This was unexpected because strains with greater than

80% DNA homology usually produce identical polyacrylamide gel patterns. This procedure may be useful for primary identification when reliable reference patterns are available.

B. Genetics of *Clostridium botulinum* Toxin Production

The genetics of *C. botulinum* toxin production has been described as being bacteriophage-mediated only in types C and D (Inoue and Iida, 1970; Eklund et al., 1971; Eklund et al., 1972; Eklund and Poysky, 1974). The genetics of toxin production in other *C. botulinum* types has not yet been reported.

C. botulinum types C and D become nontoxigenic when cured of their specific bacteriophage and again become toxigenic when reinfected (Inoue and Iida, 1970; Eklund et al., 1972). Bacteriophage involvement in the interconversion of toxigenic *C. botulinum* types C and D (Eklund and Poysky, 1974) and *C. botulinum* type C and *Clostridium novyi* type A (Eklund et al., 1974) have also been reported, proving that genes coding for toxin production in *C. botulinum* may not be entirely chromosomally linked.

Bacteriophage have been isolated from *C. botulinum* types A, B, E, and F (Inoue and Iida, 1968; Eklund et al., 1969; Takumi et al., 1980a; Takumi et al., 1980b; Kinouchi et al., 1981), but their relationship to toxinogenesis has not been demonstrated. Eklund and Poysky (1974) cured strains other than types C and D of their prophages, but they still remained toxic.

C. Plasmid Deoxyribonucleic Acid

A plasmid was first defined as an extrachromosomal hereditary determinant (Lederberg, 1952) but it is now generally known as an extra-

chromosomal element which is physically separate from the chromosome of the cell and is perpetuated stably in this condition (Clowes, 1972). Plasmids exist in a covalently closed circular (ccc) form and have been isolated from both prokaryotes and eukaryotes only as double-stranded DNA circles. A wide range of gram-positive and gram-negative bacteria have been found to harbor plasmids, including Escherichia coli (Rownd, 1966; Bazaral and Helinski, 1968), Yersinia sp. (Ferber and Brubaker, 1981; Portnoy et al., 1981), Bacillus sp. (Carlton and Smith, 1974), Staphylococcus sp. (O'Reilly et al., 1981) and streptococci (Klaenhammer et al., 1978).

Many phenotypic traits have been linked to plasmids. Sm̆arda (1982) states that 10 clearly different basic phenotypic classes of plasmids are known today. They are F (fertility), R (resistance), B (bacteriocin), M (metabolic paths), P (physiological control), V (virulence), T (toxicity), Ti (tumor induction in plants), D (defective phage genome), and C (cryptic).

Plasmids have been found to be important research tools because they can be easily manipulated for the study of structure and function of genetic material. They are also used as vectors for eukaryotic genes in the growing field of genetic engineering. Recently, the hypothesis that plasmids are autonomous organisms forming the lowermost element in the hierarchy of life was proposed (Sm̆arda, 1982). This hypothesis was based on the fact that each plasmid regulates its own replication, can be involved in genetic exchange via conjugation or transduction in bacteria, and most importantly, carry genes that enable them to survive, to reproduce and to spread horizontally regardless of the fate of their

host cell and their host species. In this respect, plasmids are also important tools in the study of bacterial evolution.

D. Plasmids in Clostridium Species

The recent interest in plasmids in bacteria has led to the examination of a wide variety of microorganisms for the presence of plasmids. The clostridia, like many other bacterial species, were found to carry plasmids in a few different species. Some of these plasmids have known functions while others are either cryptic or need to be studied further.

The plasmids of Clostridium perfringens have probably been the most extensively studied of the clostridia. C. perfringens plasmids have been found to code for functions such as bacteriocin (Brefort et al., 1978), perfringocin (Mihelc et al., 1978), multiple antibiotic resistance (Sebald et al., 1975), and caseinase production (Blaschek and Solberg, 1981). Other C. perfringens strains contain plasmids which are phenotypically cryptic (Rokos et al., 1978; Rood et al., 1978a; Rood et al., 1978b; Duncan et al., 1978; Solberg et al., 1981).

Clostridium difficile, a bacterium recently linked to antibiotic-associated diarrhea, has been found to contain plasmid DNA and be resistant to tetracycline, but no connection between the two could be found (Smith et al., 1981). Clostridium cochlearium T-2 contains a plasmid that determines mercury resistance (Pan-Hou and Imura, 1981; Pan-Hou et al., 1981). Four of seven strains of Clostridium butyricum examined by Minton and Morris (1981) contained a common plasmid with no known function. Of the toxic clostridia, C. novyi type A (Schallehn and Krämer, 1981), C. tetani (Laird et al., 1980), and C. botulinum (Scott and Duncan, 1978) are known to carry plasmids. The presence of a single

large plasmid in C. tetani which was absent in nontoxigenic strains, led Laird et al. (1980) to believe that toxinogenicity is probably extrachromosomally linked. Plasmids were detected in both toxigenic and nontoxigenic strains of C. novyi type A (Schallehn and Krämer, 1981) and C. botulinum type E (Scott and Duncan, 1978), but only speculation about their being responsible for toxin production was made in both cases. The only toxic type A strain of C. botulinum screened by Scott and Duncan (1978) contained two plasmids.

E. Plasmid DNA Isolation

Bacterial plasmids are known to exist as ccc duplex DNA's. Because of their physiochemical properties they can be separated from linear DNA or open circular (oc) DNA. Plasmids differ from oc and linear DNA in their resistance to denaturation, sedimentation in sucrose gradients, buoyant density in ethidium bromide-cesium chloride gradients, and mobility in agarose gels (Broda, 1979).

Marmur (1961) described one of the earlier general isolations of biologically active DNA from several microorganisms. The bacteria were lysed with sodium dodecyl sulfate (SDS), but only fragments in excess of 6 megadaltons (Mdal) were obtained. Covalently closed circular DNA was isolated from HeLa cells by cell lysis in SDS and subsequent buoyant density centrifugation in the presence of intercalating dyes (Radloff et al., 1967). The buoyant density of a closed circular DNA-dye complex at saturation is greater than that of a linear or nicked circular DNA-dye complex, so gradient centrifugation provides separation. The isolation of polyoma DNA from infected mouse cell cultures, which was in a ccc configuration, was described by Hirt (1967). The preferential precipi-

tation of undegraded cellular DNA in the presence of SDS and sodium chloride aided in the isolation of ccc DNA from the chromosomal fragments.

In his description of the isolation and configuration of a bacterial sex factor from Proteus mirabilis, Hickson et al. (1967) used a series of techniques to try to get a "cleaner" plasmid preparation. This procedure included lysozyme and RNAse treatment of the cells with the addition of ethylendiamine tetraacetic acid (EDTA). This step induced the formation of spheroplasts in a sucrose buffer in the presence of a chelating agent. Cellular lysis was accomplished with SDS for the complete release of plasmids from the cell and its components. Repeated phenol extractions and dialysis removed proteins and other low molecular weight contaminants. Dye-buoyant density gradients and electron micrographs were used to analyze and confirm the circular DNA configuration of this bacterial sex factor. These milder conditions are still used in many modern plasmid isolation techniques.

The cleared lysate technique was first described by Clewell and Helinski (1969) when they isolated the colicinogenic factor E_1 from Brij 58 (mild detergent) lysates of E. coli. A plasmid enriched supernatant fluid (called the cleared lysate) was the result of cell lysis followed by centrifugation. About 95% of the chromosomal DNA was pelleted after the centrifugation. The cleared lysates could be further purified in sucrose gradients or dye-buoyant density equilibrium gradients.

The general method for the isolation of plasmid DNA used by Guerry et al. (1973) involved lysing the plasmid-containing cells in the same manner as Hickson et al. (1967) to release cell contents. When the sodium chloride concentration was increased to a final concentration of

1 molar, cellular DNA precipitated out as previously noted by Hirt (1967). A low-speed clearing centrifugation removed essentially all (>99%) of the chromosomal DNA, but left plasmid DNA in this cleared lysate.

Hymphreys et al. (1975) described the concentration of plasmid DNA from salt-precipitated cleared lysates in polyethylene glycol (PEG), but this procedure still required lengthy ultracentrifugation. Precipitation of cleared lysates in cold ethanol enabled Meyers et al. (1976) to eliminate the need for the time consuming gradients. DNA precipitates were suspended in a small volume of buffer and were analyzed immediately in agarose gels. The migration of the DNA concentrates in the agarose gels provided information on the number and molecular weights of plasmids in test organisms.

Large plasmids (>100 Md) have been isolated from a number of bacterial species. Currier and Nester (1976), Hansen and Olsen (1978), and Casse et al. (1979) all utilized the ability of ccc DNA to resist denaturation in alkaline conditions for the isolation of large plasmids. Since two strands of plasmid ccc DNA remain joined as interlocking rings at increased pH's, they renature much more efficiently than plasmid oc DNA and chromosomal DNA (Broda, 1979). Each of these procedures started with a cleared lysate which was prepared with a method modified for the specific bacterium used. Currier and Nester (1976) used ethanol precipitation and a cesium chloride gradient to characterize plasmid DNA in Agrobacterium. Direct agarose gel electrophoresis of plasmid DNA concentrated in PEG (Hansen and Olsen, 1978) or ethanol (Casse et al., 1979) was also used to detect large plasmids in a number of organisms.

Most recently, rapid screening procedures for the detection of plasmids have been reported. Leblanc and Lee (1979) described a procedure for screening plasmids in all species of streptococci. This method can be used for screening several hundred isolates in a short period of time. The rapid method of Kado and Liu (1981) can be used on a variety of bacteria to obtain plasmid DNA that can be used directly in nick translation, restriction endonuclease analysis, transformation, and DNA cloning experiments.

The procedures being reported in current journals are mostly combinations and modifications of the techniques outlined in this review. Each time a new system is identified, changes in the procedures may be necessary. The new procedure will probably be made of parts of older ones to get the anticipated results.

These plasmid isolation techniques have evolved into useful procedures to isolate and characterize plasmid DNA and the uses of plasmids as vectors and research tools will become more widespread as their functions become more fully understood. There are still many prokaryotic and eukaryotic systems to be examined.

MATERIALS AND METHODS

A. Chemical and Bacteriological Media

Bacteriological enzyme TBL-1 of Takeda Chemical Industries, Ltd., Japan, was obtained from Dr. J. L. Johnson of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA. Medium electroendoosmosis (ME) agarose was purchased from Marine Colloids, FMC Corp., Rockland, ME. Dextrose, sucrose, and sodium dodecyl sulfate (SDS) were procured from Fisher Scientific Chemical Co., Raleigh, NC. Tris (hydroxymethyl) aminomethane (Tris), Tris hydrochloride (Tris-HCl), ethylenediamine tetraacetic acid (EDTA), and disodium EDTA, were purchased from Sigma Chemical Co., St. Louis, MO. Bacteriological ingredients Trypticase peptone, peptone, yeast extract, Trypticase Soy Broth (TSB), and Trypticase Soy Agar (TSA), were obtained from BBL, (Cockeysville, MD), beef extract from Gibco Diagnostics, (Madison, WI), and tryptone from Difco Laboratories, (Detroit, MI). *C. botulinum* type A antitoxin was obtained from the Centers for Disease Control, Atlanta, GA.

B. Maintenance and Identification of Test Organisms

Clostridium sporogenes strains VPI 1963, 2669, 4131C, and 6054D, and *Escherichia coli* strain V517 (Macrina et al., 1978) were obtained from Dr. J. L. Johnson of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA. *Erwinia stewartii* strain SW_2 (Bradshaw-Rouse et al., 1981; Gantotti and Beer, 1982) was obtained from Dr. D. Coplin (Department of Plant Pathology, Ohio Agriculture Research and Development Center, Wooster, OH). *C. botulinum* type A strains 62, 52, 36, 33, and 10755, and *C. sporogenes* strain 3679

were from the Food Science culture collection. Strain number 77 was received as a C. botulinum type A strain from Dr. L. N. Christiansen (Swift & Co., Oak Brook, IL), but is renamed C. sporogenes 77 in this study due to its lack of toxicity in the mouse bioassay (AOAC, 1978) and its performance in other bacteriological identification tests.

Stock cultures of C. botulinum type A and C. sporogenes were maintained in prereduced chopped meat broth at room temperature. Standard plasmid containing strains of E. coli and E. stewartii were stored aerobically on TSA slants at 4°C and in deep stabs of L-agar (Miller, 1972) at room temperature respectively. All bacterial stocks were transferred periodically to a fresh medium.

Phenotypic characteristic screening (Holdeman et al., 1977), toxin typing (AOAC, 1978), and polyacrylamide gel electrophoresis (PAGE) of soluble cellular proteins (Cato et al., 1982) were performed on all test strains to ensure purity and identity.

C. Preparation of Media for Growth Studies

Prereduced peptone-yeast extract-glucose (PYG) medium containing .5% peptone, .5% Trypticase peptone, 1% yeast extract, and 1% glucose, and chopped meat broth were prepared as described by Holdeman et al. (1977). Both media were prepared to have a final pH of 7.0. Oxygen was driven off and the ingredients were partially reduced by boiling. The media were further reduced with added cysteine. Media were kept oxygen-free by flushing with anaerobe grade carbon dioxide and keeping tubes and flasks tightly stoppered.

All anaerobic organisms were transferred under oxygen-free gas using the VPI Anaerobic Culture System (Bellco Glass Inc., Vineland, NJ).

Antibiotic medium No. 3 contained .15% beef extract, .15% yeast extract, .5% peptone, .1% glucose, .35% sodium chloride, 20 mM dipotassium phosphate, and 9 mM monopotassium phosphate as described by Difco Laboratories (Detroit, MI). L-agar consisted of 1% tryptone, .5% yeast extract, .05% sodium chloride, 0.2% glucose, and 1.5% agar and was prepared as described by Miller (1972).

D. Preparation of Inoculum and Growth Studies

Actively growing inocula of C. botulinum type A and C. sporogenes were initiated by transferring 5-10 drops of stock culture to 10 ml of prereduced PYG broth followed by incubation for 10-12 hours at 37°C. A 0.3 ml portion of the freshly grown culture was transferred to another tube containing 10 ml of prereduced PYG broth and incubated for 10 hours at 37°C. This culture was used as an inoculum in the subsequent growth studies because it contained many actively growing cells.

Prereduced PYG (350 ml) was prepared in 500 ml Nephlo culture flasks (Bellco Glass Inc., Vineland, NJ) for the growth studies. A 1% (v/v) inoculum of freshly grown cells was added to culture flasks containing the growth medium and incubated at 37°C in a shaker water bath (Fermentation Design Inc., Allentown, PA) at 150 RPM. The flasks were closed with a two-holed stopper to keep them anaerobic. Glass tubing was fitted into the stopper holes and covered with clamped vacuum tubing to prevent gas exchange during the early stages of growth. When positive pressure from gas production increased, one side of the tubing was vented. Continuous gas flow out of the flask prevented oxygen from entering during the latter stages of growth.

Growth curves were constructed from hourly optical density (OD)

readings using a Bausch and Lomb Spectronic 20 (600 nm) for 12 hours. At three pre-determined stages of the growth cycle, 40 ml of culture were aseptically removed with a sterile pipette for plasmid isolation.

Aerobic organisms were also transferred twice when they reached middle to late logarithmic growth in their respective media, to obtain actively growing cells before they were used as inocula. E. coli growth was initiated with a 1% (v/v) inoculum into 50 ml of Antibiotic Medium No. 3 and incubated aerobically at 37°C in a shaker water bath. E. stewartii SW_2 growth was started similarly in 50 ml of TSB at 32°C. The cells were harvested at their middle to late logarithmic stages of growth which corresponded to OD readings (600 nm) of approximately .42 for E. coli V517 and .25 for E. stewartii SW_2.

E. Isolation of Plasmids from C. botulinum type A and C. sporogenes

The procedure used in this study was a modification of the methods described by Casse et al. (1979), Leblanc and Lee (1979), and Johnson (1982). Each 40 ml of C. botulinum type A or C. sporogenes culture was pelleted by centrifugation at 12,000 x g for 30 min. (4°C). The cell pellets were suspended in 0.5 ml Tris-sucrose buffer (50 mM Tris-HCl, pH 8.0 containing 25% w/v sucrose) with a sterile rubber policeman and transferred to sterile 15 ml Corex high-speed centrifuge tubes. TBL-1 enzyme, 10 µl of a 6.25 mg/ml solution, was added to the tubes containing the suspended cell pellets to obtain a final concentration of 300 units/ml. TBL-1 has a specific activity for bacteriolysis of 2,400 units/ml. The cell and enzyme suspension was gently mixed by tapping the tube and then incubated in a water bath at 37°C for 10 min.

Cells were lysed by adding 3.8 ml of freshly prepared lysing buffer

(4% w/v sodium dodecyl sulfate in 50 mM Tris, 20 mM EDTA free acid, pH 12.45). Gentle inversion of the tubes gave rise to a clear, viscous suspension of lysed cells. To bring the pH back towards neutrality, 0.4 ml of 2 M Tris (pH 7.0) was added and again gently mixed by inversion. To obtain a final NaCl concentration of 1 M, 1.2 ml of a 5 M NaCl solution was added and mixed until a thick white precipitate formed. The lysate was cooled on ice for one hour to aid in the precipitation of the higher molecular weight chromosomal DNA. After centrifugation at 17,000 x g for 30 min. (4°C), the supernatant fluid was poured into a clean Corex tube and mixed with 5 ml phenol, which was previously equilibrated with an equal volume of 0.5 M NaCl, and the precipitate was discarded. The suspension was centrifuged at 5,000 x g for 10 min. (4°C) to break the emulsion and the aqueous phase was collected with a sterile 5 ml pipette. The volume was doubled with cold ethanol (which was previously cooled to -20°C) and stored overnight in the freezer to precipitate the nucleic acids. The precipitate was pelleted by centrifuging the alcohol-nucleic acid suspension at 17,000 x g for 30 min. (-10°C) and suspended in 100 µl TE buffer (0.05 M Tris, 0.02 M Na_2EDTA, pH 8.0). All DNA was stored in TE buffer at 4°C in sterile Wheaton Cryules (Wheaton Scientific, Raleigh, NC).

Plasmids were isolated from *E. coli* and *E. stewartii* as described above, but without the addition of the bacteriolytic enzyme TBL-1.

F. Agarose Gel Electrophoresis

DNA was subjected to electrophoresis in horizontal slab gels (0.3 x 20 x 25 cm) of 0.7% ME agarose on a BRL horizontal system for submerged electrophoresis (Bethesda Research Laboratories, Inc., Rockville, MD).

Agarose, 1.22 g, was weighed into a 250 ml Erlenmeyer flask and the weight was brought up to 175 g with E buffer (40 mM Tris, 2 mM Na_2EDTA, pH 7.9 with glacial acetic acid). The agarose was dissolved by boiling in a microwave oven and cooled at room temperature to 50-60°C before pouring. The gel was allowed to solidify at room temperature for 30 min. Well slots were formed with either a 3 mm (10 well, Bio Rad Laboratories, Richmond, CA) or a 1 mm (20 well, Bethesda Research Laboratories Inc., Rockville, MD) comb.

The wells were loaded with 40 or 20 µl of DNA suspension and 5 or 2 µl of tracking dye (0.01% w/v bromophenol blue, 5% w/v sodium dodecyl sulfate, 50% v/v glycerol) depending on the comb used. The gels were run at 150 volts constant current from a Buchler Power Supply (Buchler Instruments Inc., Ft. Lee, NJ) for 15 min. or until the track dye entered the gel. The power was stopped, the gel was submerged in E buffer, and the electrophoresis was continued at 100 volts (32 mA) for about 12 hours. After 12 hours, the tracking dye usually came off the end of the gel. The gel slabs were stained in 5 mg/ml ethidium bromide in distilled water for 30 min., destained for 10 min. in distilled water, and visualized on an ultraviolet transilluminator (Ultraviolet Products Inc., San Gabriel, CA). Photographs were taken with Polaroid type 55 positive/negative film through a #23A red filter. A Polaroid film holder #500 was used with a Polaroid Pathfinder Land camera 110 lens and bellows.

G. Plasmid Molecular Weight Determination

Molecular weight estimates for test strain plasmids were extrapolated from standard curves constructed from the migration distances of plasmids of known molecular weight. Plasmids from *E. coli* strain V517 were used

as one set of molecular weight markers. This bacterium contains 8 plasmids with molecular weights of 35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, and 1.4 Mdal. (Macrina et al., 1978). The logarithm of each migration distance (measured with a caliper) in 0.7% agarose plotted against the logarithm of the known molecular weight for each plasmid produced a straight line standard curve. Linear regression analysis was used to obtain a "best fit" line for the points. E. coli V517 markers were run next to test strain DNA and curves were constructed for all agarose gels. Erwinia stewartii SW_2 markers were used to confirm the migration of the E. coli plasmids in one gel. E. stewartii SW_2 contains 12 plasmids of 178, 69.8, 51.6, 49.2, 43.3, 34.5, 33.0, 29.5, 23.2, 16.9, 8.8, and 2.8 Mdal. (Gantotti and Beer, 1982). At least three measurements were taken for each test strain plasmid.

RESULTS AND DISCUSSION

A. Identification of Test Organisms

Phenotypic characteristic screening included Gram's staining, microscopic and macroscopic examination, gas chromatography for analysis of acid and alcohol products, antibiotic susceptibility, and a number of other biochemical tests as described by Holdeman et al. (1977). Toxin testing and typing was performed in a mouse bioassay (AOAC, 1978). This phenotype screening and the toxin typing confirmed the identity of all test strains used in this study.

Polyacrylamide gel electrophoresis (PAGE) of test strain soluble cellular proteins (Cato et al., 1982) agreed with available reference banding patterns for all C. botulinum type A strains and all but one C. sporogenes strain (Figure 1). C. sporogenes strain 77 (Figure 1, lane A) did not produce the same general protein pattern in the PAGE as any of the other C. sporogenes or C. botulinum strains, but was identified as a C. sporogenes after gas chromatography, biochemical, and toxicological analysis, and therefore remained as such in this study. C. sporogenes 77 was nontoxic. All other C. sporogenes strains (Figure 1, lanes G-L) produced the characteristic protein bands at 4 mm and 62 mm in the PAGE which were absent in the toxic strains of C. botulinum type A (Figure 1, lanes B-F).

B. Growth Studies

Growth curves of C. botulinum type A strains (Figure 2), plasmid-containing strains of C. sporogenes (Figure 3), and non-plasmid containing strains of C. sporogenes (Figure 4), show the time for cultures to reach

Figure 1. Polyacrylamide gel electrophoresis of soluble cellular proteins (Cato et al., 1982) of C. sporogenes 77 (lane A), C. botulinum 52A (lane B), C. botulinum 62A (lane C), C. botulinum 36A (lane D), C. botulinum 10755A (lane E), C. botulinum 33A (lane F), C. sporogenes 6054D (lane G), C. sporogenes 2669 (lane H), C. sporogenes 3679 (lane I), C. sporogenes 2022 (lane J), C. sporogenes 4131C (lane K), and C. sporogenes 1963 (lane L).

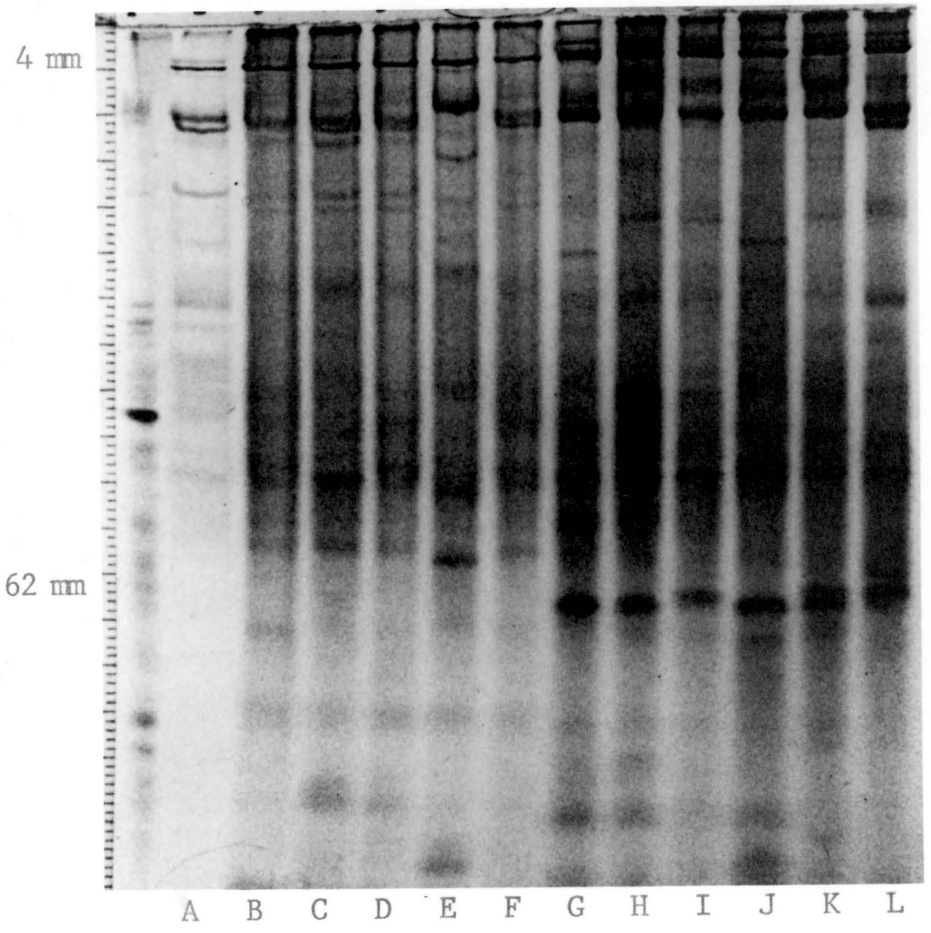

4 mm
62 mm
A
B
C
D
E
F
G
H
I
J
K
L

Figure 2. Growth of Clostridium botulinum type A strains in prereduced peptone-yeast extract-glucose broth (pH 7.0) at 37°C.

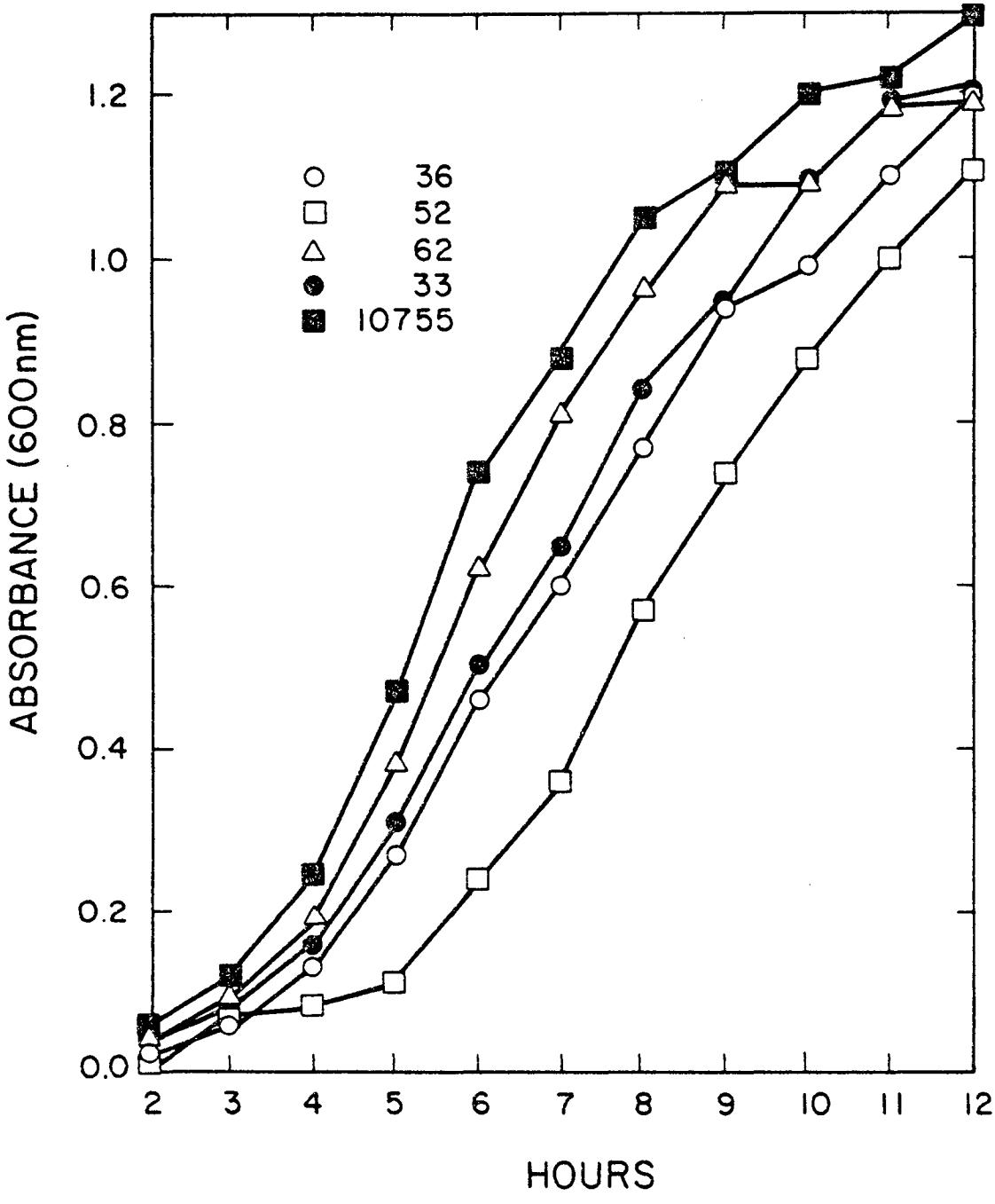

ABSORBANCE (600nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
36
52
62
33
10755
2
3
4
5
6
7
8
9
10
11
12
HOURS

Figure 3. Growth of plasmid-containing strains of Clostridium sporogenes in prereduced peptone-yeast extract-glucose broth (pH 7.0) at 37°C.

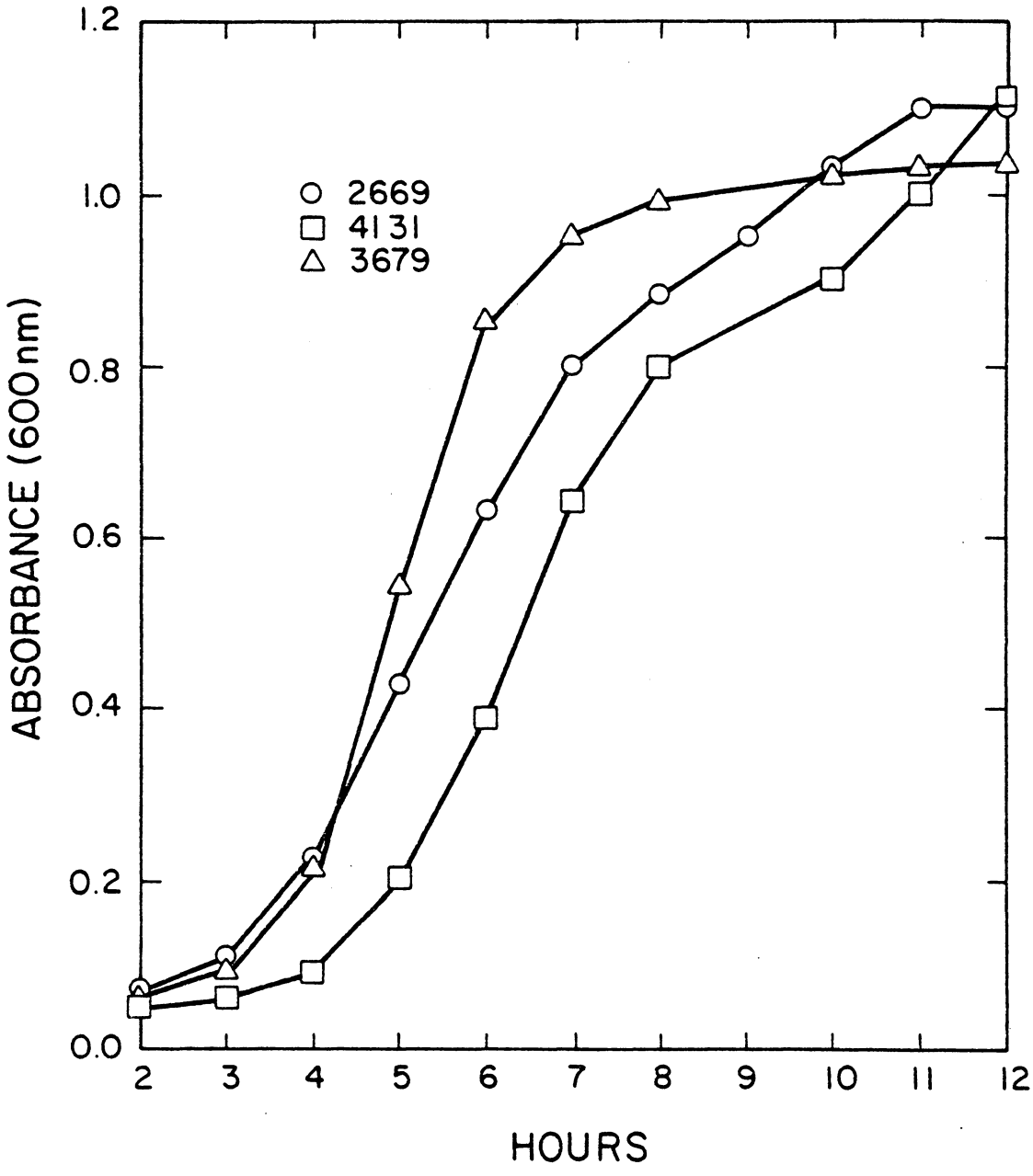
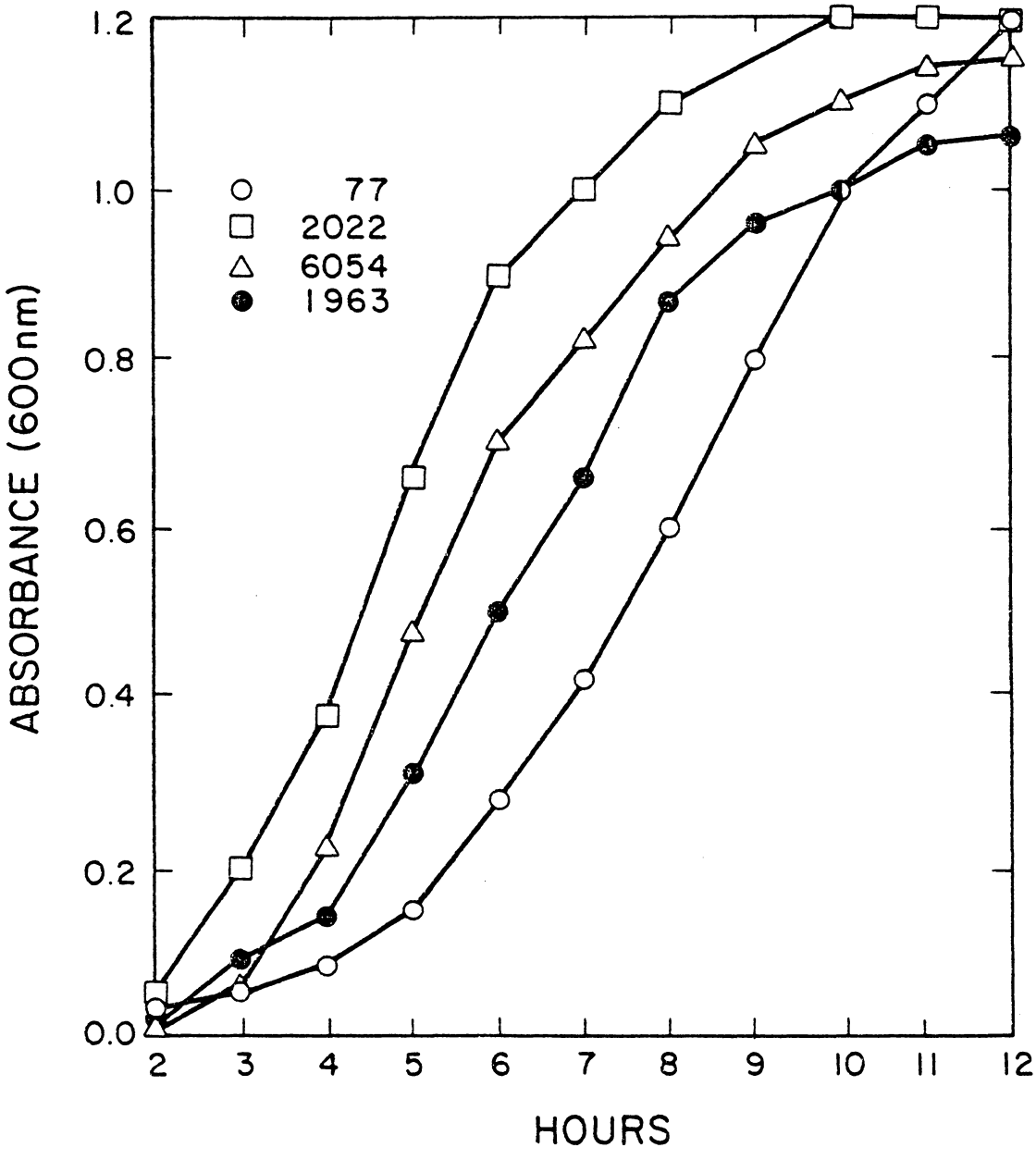

1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE (600nm)
2669
4131
3679
2
3
4
5
6
7
8
9
10
11
12
HOURS

Figure 4. Growth of non-plasmid containing Clostridium sporogenes strains in prereduced peptone-yeast extract-glucose broth (pH 7.0) at 37°C.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE (600nm)
77
2022
6054
1963
2
3
4
5
6
7
8
9
10
11
12
HOURS

the middle to late log growth phase which was important in the plasmid isolation procedure. This growth stage was evident when OD readings (600 nm) were in the 0.8 to 1.0 range for all C. sporogenes and C. botulinum type A strains. The five C. botulinum type A strains needed a range of 6-10 hours to reach this growth stage (OD), while all C. sporogenes strains obtained this OD range in a more consistent 7-9 hours. Optical density was recorded for 12 hours to observe when the stationary phase occurred for all test strains.

C. Plasmid DNA Isolation

1. Plasmid Extraction

The procedure which led to the isolation of the plasmids involved the formation of spheroplasts, lysis of the cells under alkaline conditions, preferential precipitation and removal of the bulk of the chromosomal DNA, extraction of protein, and the precipitation and concentration of the plasmid DNA, in that order. Each step in this sequence is important and therefore each will be cited and discussed.

Spheroplasts were formed by treating C. botulinum type A and C. sporogenes vegetative cells with a bacteriolytic enzyme in a hypertonic solution of buffered sucrose. As described by Galli and Hughes (1965), with C. sporogenes, and Kawata et al. (1968), with C. botulinum type A, spheroplasts in a hypertonic solution are fragile and cytoplasmic contents (which includes most plasmids) may leak out into the buffer. At this point the majority of the plasmids in the test strains were freed and separated from the cell components.

Lysis of the cells with SDS provides two functions. One is that any plasmids that may be attached to the cell wall of the bacteria will

be released (Guerry et al., 1973). The other advantage is that the chromosomal DNA is released, exposed, and denatured under the alkaline conditions. Denaturation of the chromosome occurs around pH 12.3 and the highly alkaline pH also tends to denature proteins, such as nucleases, which could degrade plasmid DNA (Casse et al., 1979). The ability of ccc DNA to resist denaturation under these conditions provides the means by which plasmid DNA is separated from linear chromosomal DNA (Broda, 1979). The remainder of the procedure involves the physical separation of the ccc DNA from the cell components and the cleaning of the plasmid preparation.

Sodium chloride, added to a final concentration of about 1 M in the presence of SDS (from lysing buffer), preferentially precipitated essentially all of the cellular DNA, leaving the plasmid DNA in the supernatant (Hirt, 1967). A low speed clearing centrifugation provided removal of the cleared lysate from the precipitated chromosomal DNA (Guerry et al., 1973).

A brief phenol extraction allowed the denaturation and extraction of any protein which did not precipitate out with the chromosomal DNA (Meyers et al., 1976). The aqueous phase was collected while the denatured protein remained at the hydrophobic-aqueous interface (Currier and Nester, 1976). This left only small fragments of linear DNA and some RNA as contaminants of the plasmid preparation.

Precipitation of the DNA in cold ethanol made it possible to concentrate the nucleic acids so that small volumes could be analyzed in agarose gels (Meyers et al., 1976). TE buffer was used as a storage solution for the DNA because the chelating properties of disodium EDTA

protected it from enzymatic degradation.

2. Cell Lysis and Culture Age

Test strains were screened for plasmids at three stages of the growth curve (early log, middle to late log, and stationary phase) to determine if culture age affects the plasmid isolation. The pre-determined culture ages corresponded to OD (600 nm) readings of approximately .6, .9, and 1.1 respectively for all test strains (Figures 2-4).

The importance of culture age in screening test strains for plasmids can be seen in a series of isolations at the three growth stages with C. botulinum 62A (Figure 5, lanes A-C) and C. sporogenes strain 2669 (Figure 6, lanes A-C). In both figures, lane A represents cells that were lysed in early log, lane B, cells in middle to late log, and lane C represents the isolation of plasmids from stationary phase cells. In Figure 5, lane A, only one of two C. botulinum 62A plasmids is easily seen and the chromosomal DNA band is minimal. In the same Figure, lane B, two plasmids are easily seen and the chromosomal DNA masks only a small area. In lane C of Figure 5, the smaller of the two plasmid bands begins to narrow and the chromosomal DNA masks a larger area. Similar results are more evident in Figure 6, lane A, where only one plasmid band and the area of chromosomal fragments are visible from C. sporogenes strain 2669. In lane B, two plasmids, one larger and one smaller than the chromosomal area, can be seen and in lane C, only the smaller plasmid is barely detected. In Figures 5 and 6, lane B seems to represent optimal plasmid detection conditions for these organisms because plasmid DNA was more easily detected and chromosomal fragments were at a minimum. Similar results were obtained for C. botulinum 33A and 36A (Figure 7).

Figure 5. Agarose gel (0.7%) electrophoresis of plasmids from C. botulinum 62A at early log (lanes A, E, H), middle to late log (lanes B, F, I), and stationary (lanes C, G, J) phase cells. Lane D is plasmids from E. coli V517, 30 µl of DNA suspension was loaded in wells A-G and 20 µl in wells H-J.

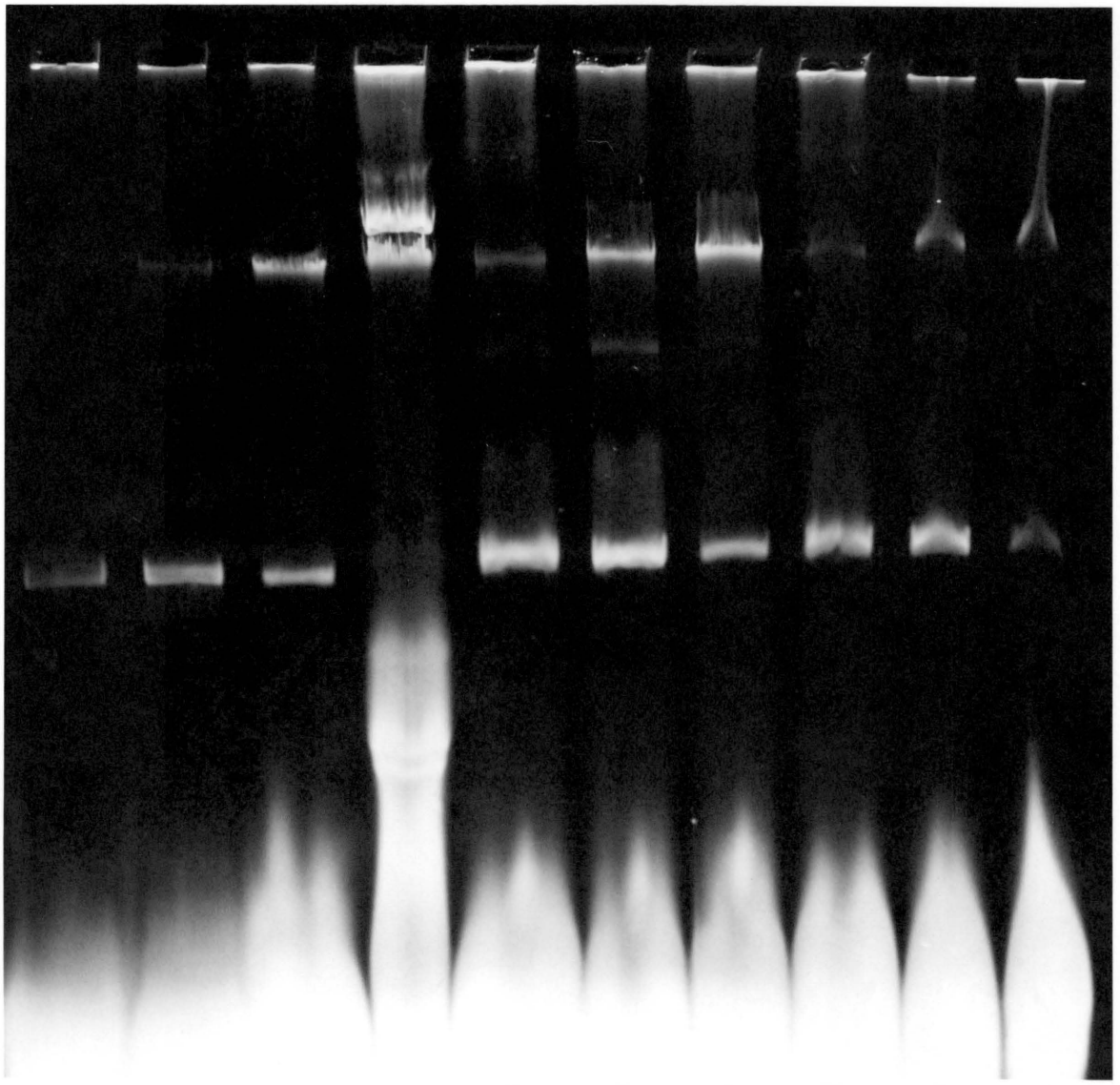

A
B
C
D
E
F
G
H
I
J

Figure 6. Agarose gel (0.7%) electrophoresis of plasmids from C. sporogenes 2669 at early log (lane A), middle to late log (lane B), stationary phase (lane C), E. coli V517 (lane D), C. sporogenes 77 (lanes E-G), E. coli V517 (lane H), C. sporogenes 2022 (lane I), and C. sporogenes 4131 (lane J). 40 µl of DNA suspension was loaded in all wells.

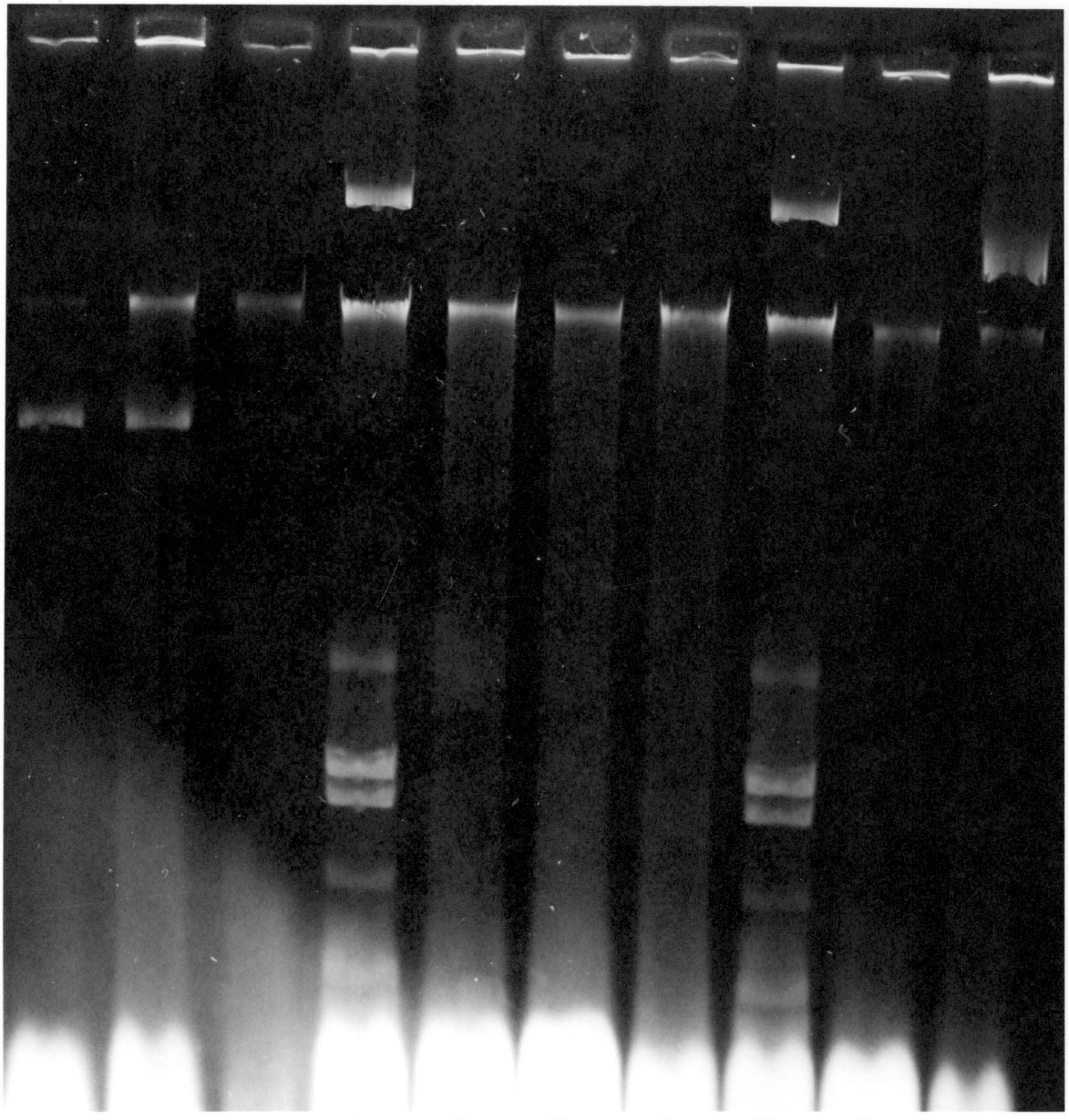
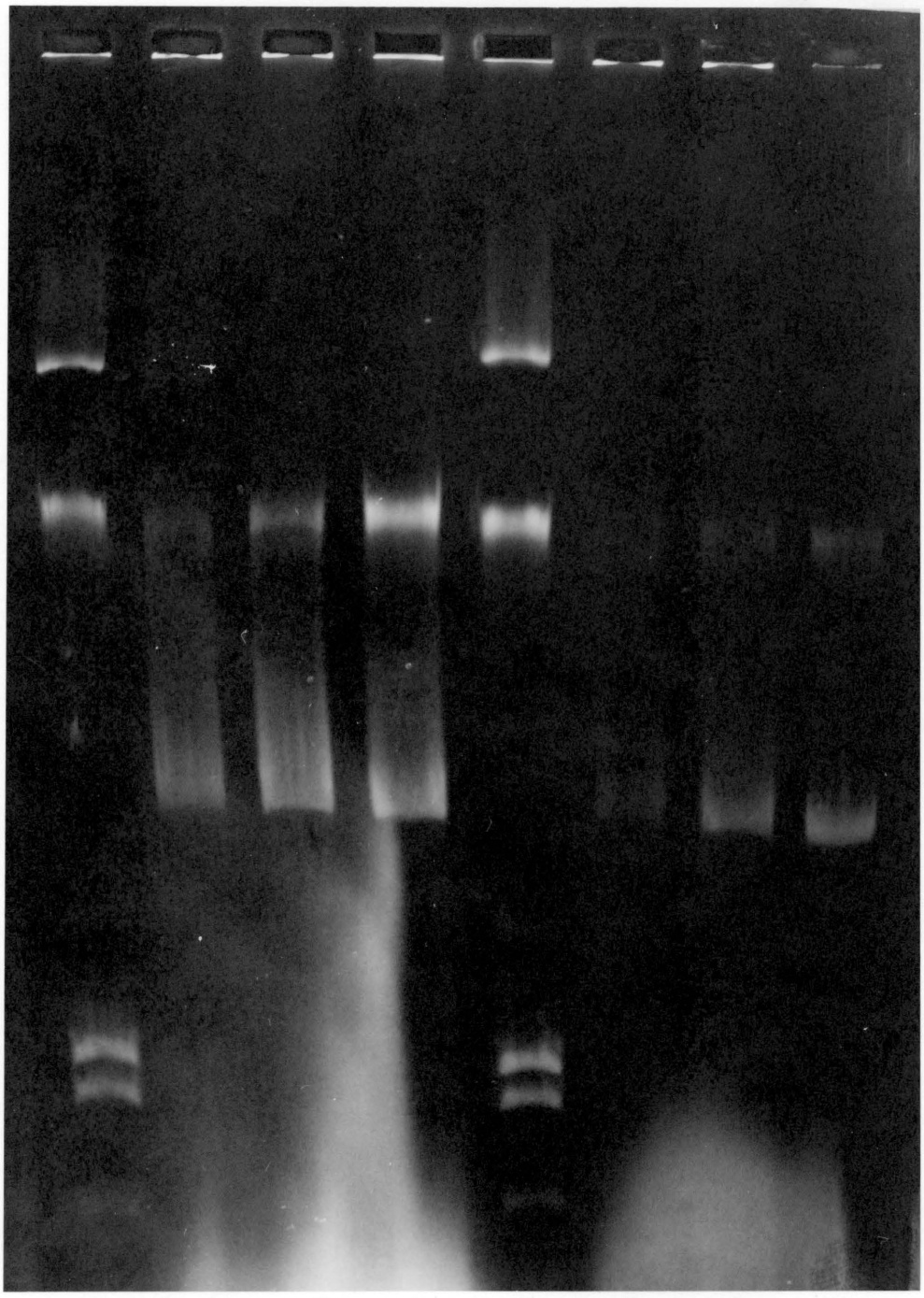

A
B
C
D
E
F
G
H
I
J

Figure 7. Agarose gel (0.7%) electrophoresis of plasmids from E. coli V517 (lane A), C. botulinum 33A at early log (lane B), middle to late log (lane C), and stationary phase (lane D), E. coli V517 (lane E), and C. botulinum 36A at early log (lane F), middle to late log (lane G), and stationary phase (lane H). 40 µl of DNA suspension was loaded to all wells.


A
B
C
D
E
F
G
H

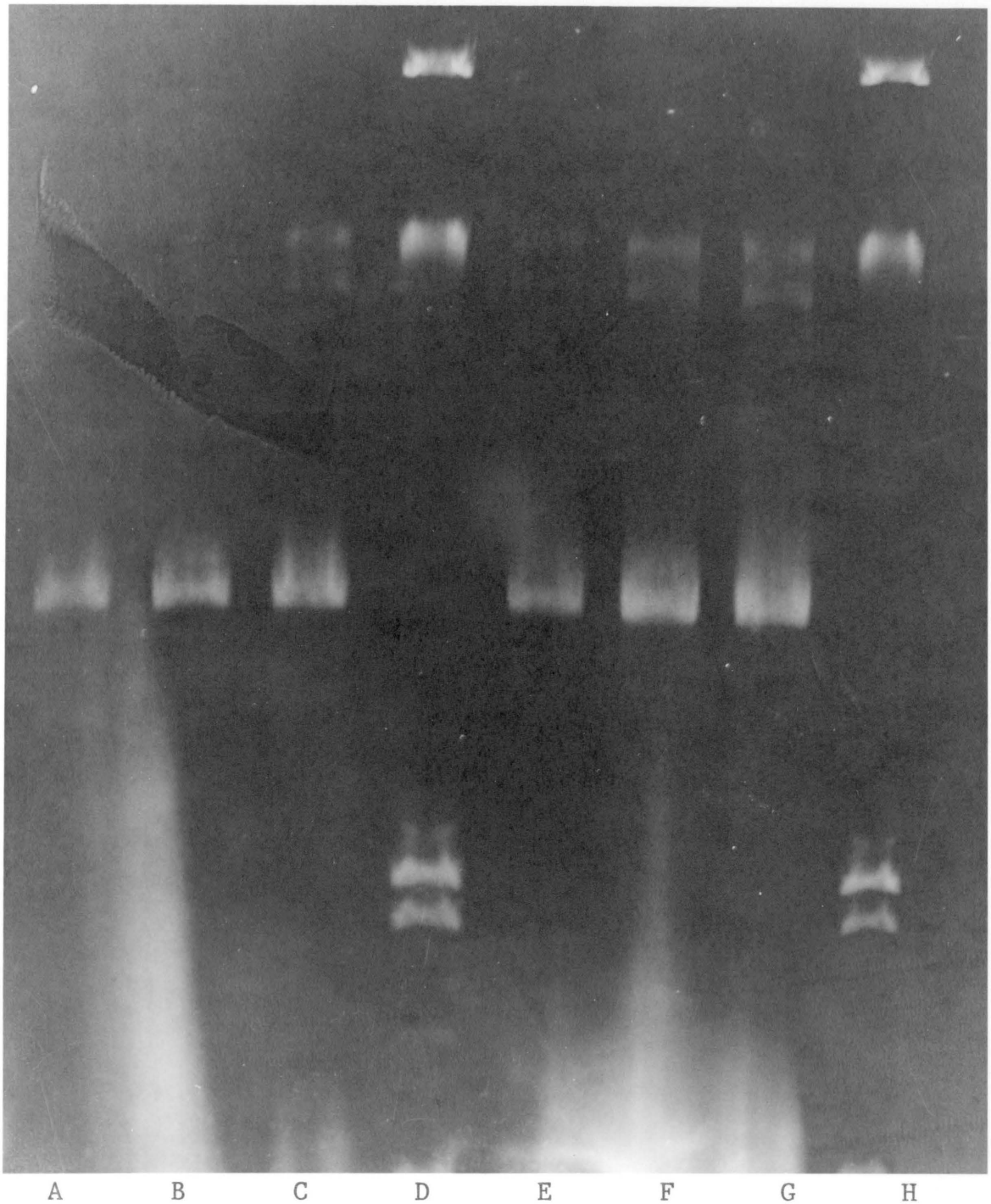
As described by Galli and Hughes (1965), the autolysis of C. sporogenes varied as the cells became older, mainly due to changes in the composition or structure of the cell wall. They noted that the organisms in buffered sucrose lyse slowly with the appearance of spheroplasts and that dilution of the sucrose leads to the greatest lysis in the youngest organisms and the least in the oldest.

A similar phenomenon could have taken place in this study when test strains were treated with a bacteriolytic enzyme and lysed in buffered sucrose during three points in their life cycle. Young vegetative cells (early log) lysed efficiently, but because of the small number of cells present (low OD = low cell density), there probably wasn't enough plasmid DNA for easy detection in the agarose gels. Efficient cell lysis was apparent when the cell suspension cleared immediately upon the addition of the lysing buffer. Cultures that had reached the stationary phase did not lyse well, which was evident by a lack of clearing when the lysing buffer was added. This incomplete lysis may have lowered the plasmid yield which would make detection more difficult. Middle to late log cultures seemed to be ideal for both lysis and plasmid detection. The vegetative cells lysed well with the addition of the lysing buffer and because of the greater number of cells, there was probably more DNA present and it was easier to detect the plasmid bands.

3. Plasmid Detection

Figures 6-13 show the agarose gel electrophoresis results of the plasmid screening of all test strains of C. botulinum type A and C. sporogenes. Agarose gels have been shown to be a simple method for detecting plasmids of various sizes in bacteria (Meyers et al., 1976).

Figure 8. Agarose gel (0.7%) electrophoresis of plasmids from C. botulinum 62A (lanes A-C), C. botulinum 52A (lanes E-G), and E. coli V517 (lanes D and H). 40 µl of DNA suspension was loaded in all wells.



A B C D E F G H

Figure 9. Agarose gel (0.7%) electrophoresis of plasmids from C. botulinum 33A (lanes A-C), C. sporogenes 6054D (lanes D-F), E. coli V517 (lane G), and C. botulinum 10755A (lanes H-J). 40 µl of DNA suspension was loaded in all wells.

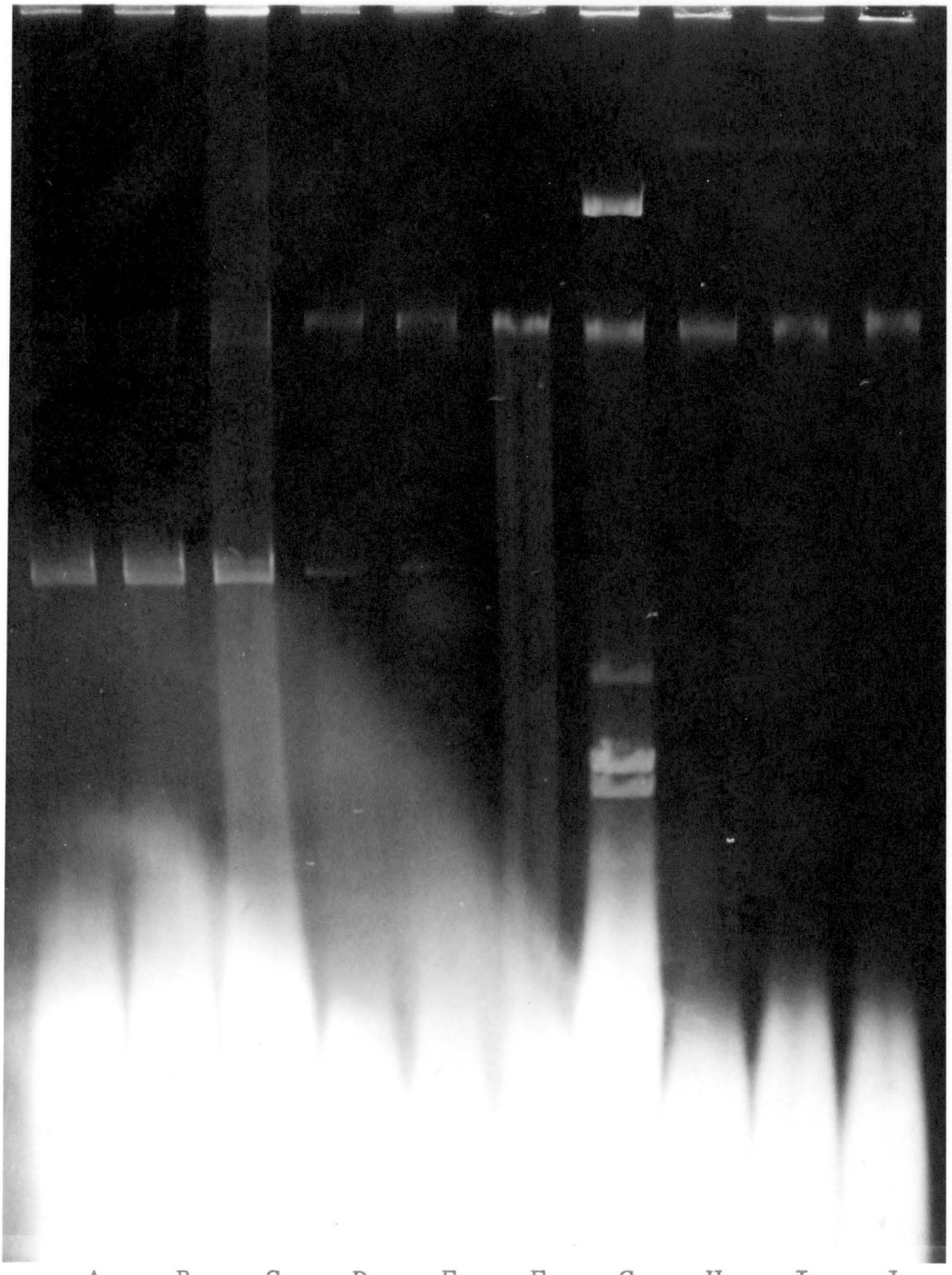

A
B
C
D
E
F
G
H
I
J

Figure 10. Agarose gel (0.7%) electrophoresis of plasmids from E. coli V517 (lanes A, E, H), C. botulinum 33A (lanes B-D), and C. sporogenes 3679 (lanes F and G). 40 µl of DNA suspension was loaded in all wells.

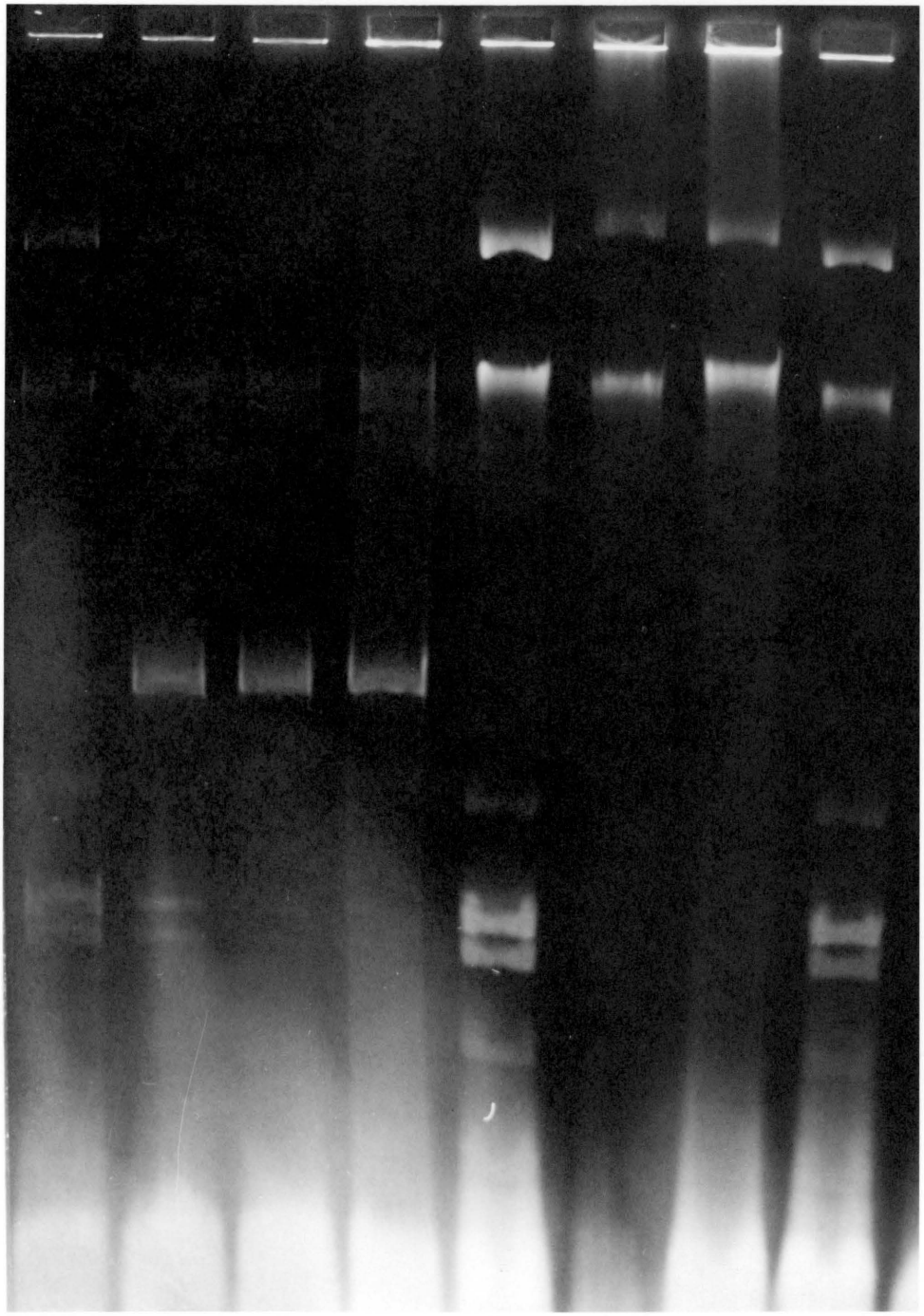

A
B
C
D
E
F
G
H

Figure 11. Agarose gel (0.7%) electrophoresis of plasmids from C. sporogenes 1963 (lanes A-C), C. sporogenes 6054D (lanes D-F), E. coli V517 (lane G), and C. botulinum 36A (lanes H-J). 40 µl of DNA suspension was loaded to all wells.

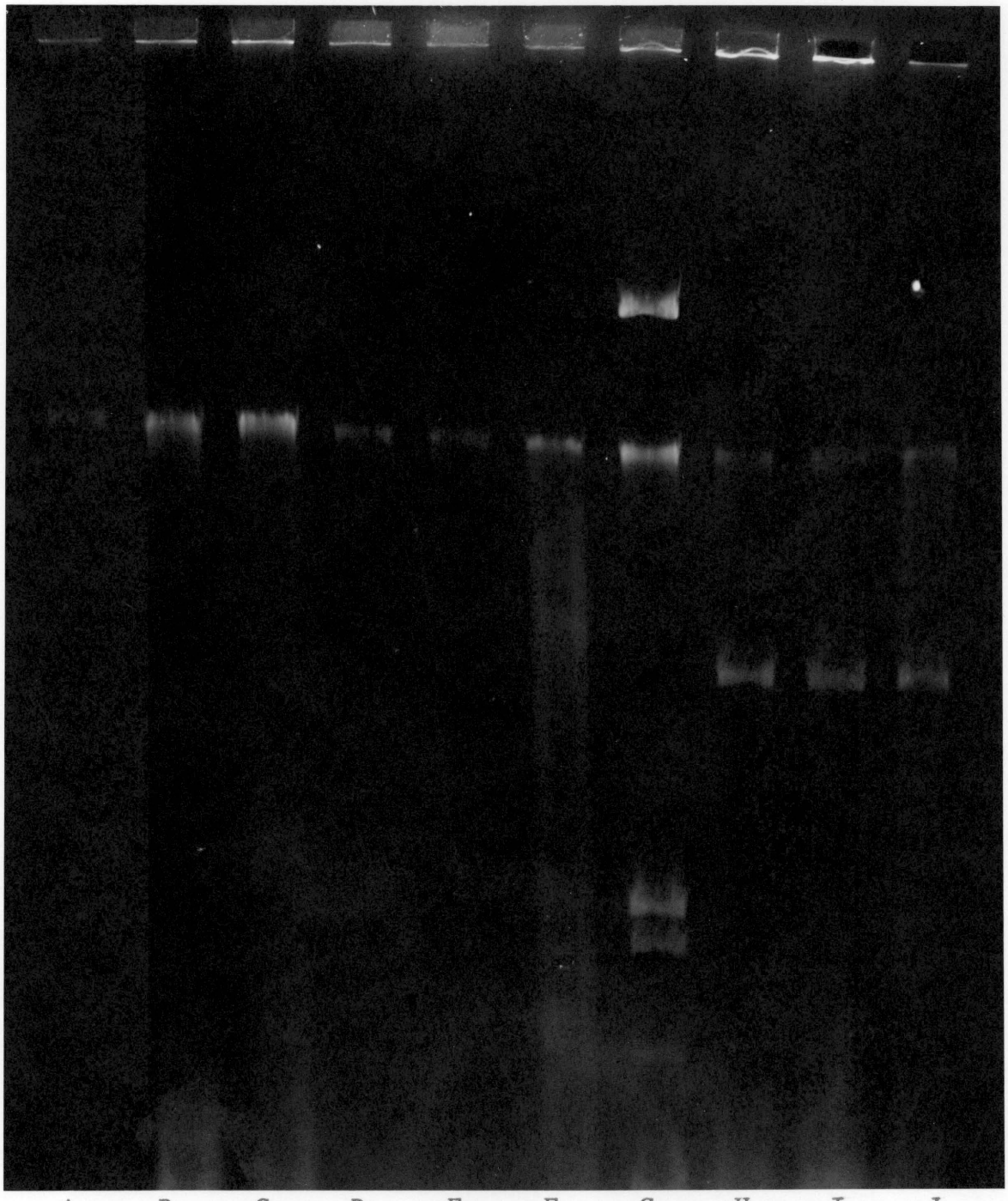

A
B
C
D
E
F
G
H
I
J

Figure 12. Agarose gel (0.7%) electrophoresis of plasmids from C. botulinum 62A (lane A), E. coli V517 (lane B and F), C. botulinum 52A (lane C), C. sporogenes 4131C (lane D), C. sporogenes 3679 (lane E), and C. sporogenes 2669 (lane G). 40 µl of DNA suspension was loaded to all wells.

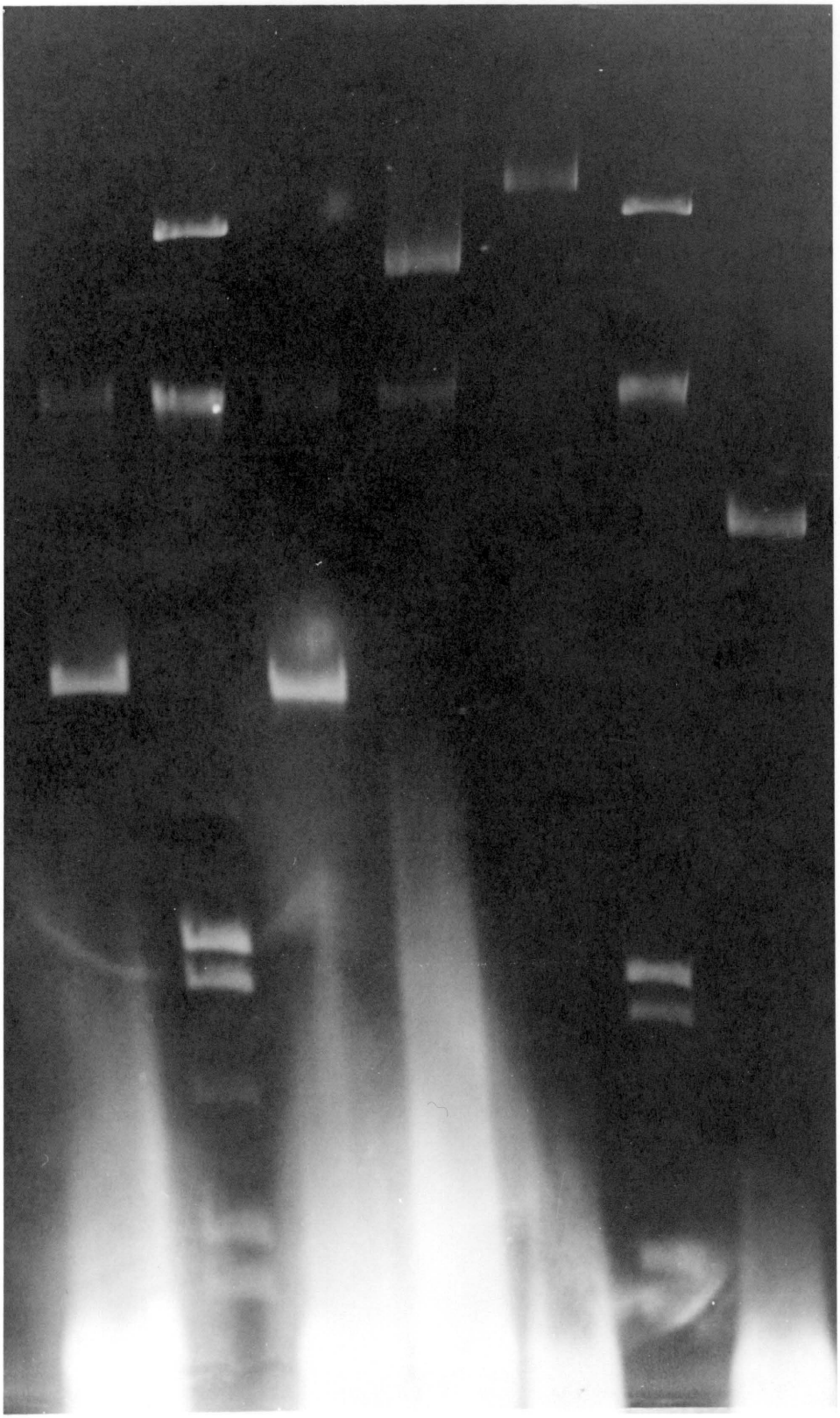
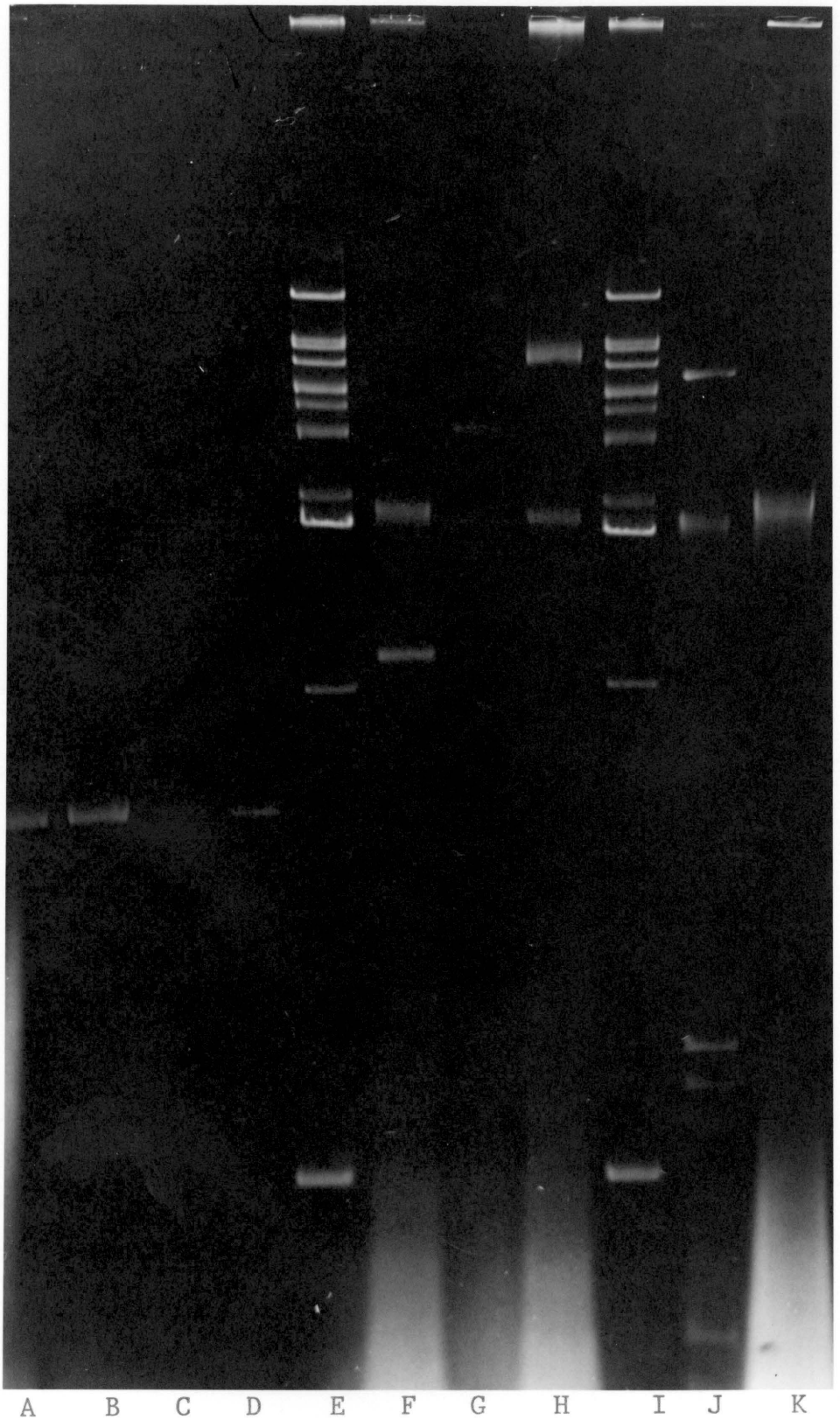

A
B
C
D
E
F
G

Figure 13. Agarose gel (0.7%) electrophoresis of plasmids from C. botulinum 62A (lane A), C. botulinum 36A (lane B), C. botulinum 52A (lane C), C. botulinum 33A (lane D), E. stewartii SW_2 (lanes E and I), C. sporogenes 2669 (lane F), C. sporogenes 4131C (lane G), C. sporogenes 3679 (lane H), E. coli V517 (lane J), and C. sporogenes 77 (lane k). 20 µl of DNA suspension was loaded in all wells.


A
B
C
D
E
F
G
H
I
J
K

This method was used in this study as the final step in the isolation and visualization of C. botulinum type A and C. sporogenes plasmids. Four out of five C. botulinum type A strains and three out of seven C. sporogenes strains contained extrachromosomal DNA which could be detected by this method. A summary of these results including molecular weight estimates for the plasmids detected is provided in Table 1.

In Figures 6-12, a 3 mm comb was used to form well slots in the gels and E. coli V517 plasmids were used as molecular weight markers. In Figure 13, a 1 mm comb was used and E. stewartii SW_2 standard plasmids were used in addition to the E. coli V517 markers.

In all of the agarose gels, chromosomal fragments migrated to a uniform area. This banding area should not be confused with those of the plasmid DNA. In agarose gels, plasmids in the ccc DNA form migrate at a rate that is inversely related to their molecular weight (Meyers et al., 1976). The smaller the plasmids, the greater the distance migrated. This distance can be used to estimate molecular weight when compared to standard plasmid preparations.

If a plasmid were to migrate the same distance as the chromosomal fragments, it would not be easily detected. This problem was sometimes encountered with the plasmid-containing strains of C. botulinum type A. The larger plasmid was occasionally masked by the chromosomal DNA because it migrated to the same general area, as can be seen in Figure 9, lanes A-C with C. botulinum 33A and Figure 11, lanes H-J with C. botulinum 36A. Only one plasmid band was visible while the other was obscured. The plasmid that was close to the chromosomal DNA was clearly observed in the original photograph for Figure 13; however, in copying the photo-

graph, this area was not clearly reproduced. In Figure 8, lanes A-C (C. botulinum 62A) and lanes E-G (C. botulinum 52A) the problem was not encountered and the 'upper' plasmid band representing the larger of the two plasmids in the toxic strains can be seen just below the chromosomal DNA band. Complete removal of the chromosomal fragments is necessary to get the best results. Both plasmids for all plasmid-containing C. botulinum test strains were observed in the original photograph for Figure 13, lanes A-D.

C. sporogenes strains in which one plasmid was detected included strain 4131C (Figure 6, lane J), and strain 3679 (Figure 10, lanes F-G). The only C. sporogenes which produced two plasmid bands was strain 2669 which can be seen in Figure 6, lanes A-C.

Non-plasmid containing test strains only reveal chromosomal DNA fragments in the agarose gels. C. sporogenes test strains in this group included strains 77 (Figure 6, lanes E-G), 2022 (Figure 6, lane I), 6054D (Figure 9, lanes D-F and Figure 11, lanes D-F), and 1963 (Figure 11, lanes A-C). The only C. botulinum type A strain in which no plasmids were detected was strain 10755A (Figure 9, lanes H-J).

Figure 12 shows the plasmids of two C. botulinum strains (62A and 52A) and all of the C. sporogenes strains in which plasmids were detected. All plasmid-containing test strains are shown in Figure 13. These gels also show the size relationships of the extrachromosomal DNA detected in C. botulinum type A and C. sporogenes test strains.

Although no plasmids were detected in one of the five C. botulinum type A strains, toxin production may still be plasmid-mediated. In four of the toxic strains, two common plasmids were detected which may contain

the genes that code for toxin production. They need to be studied further in order to determine if they are involved in toxin production or some other function. In C. botulinum 10755A, it is possible that one or both of these extrachromosomal elements became integrated into the chromosome or that a transposable element was involved in transfering toxicity to the chromosome.

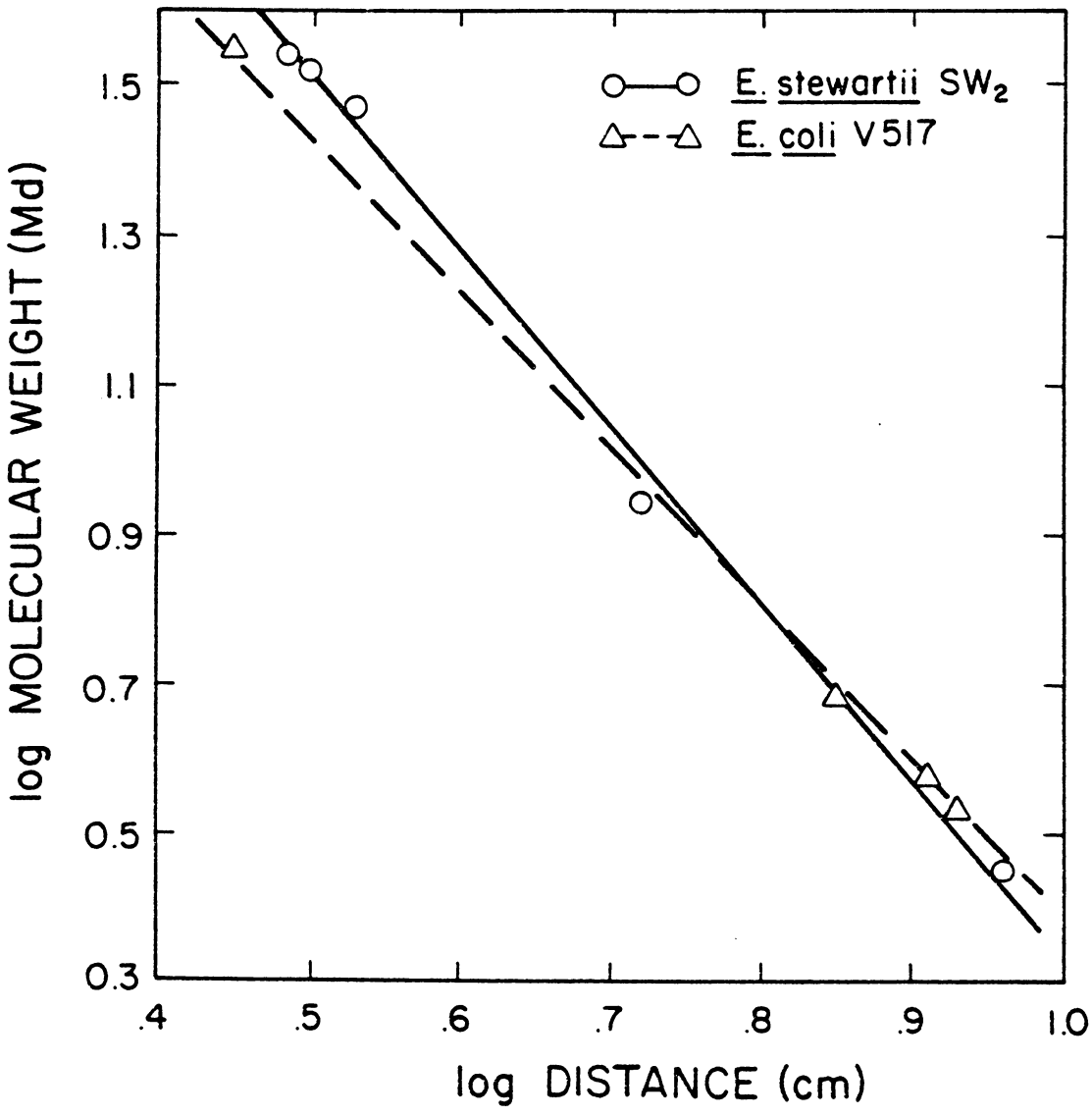
Plasmids, sometimes multiple plasmids, have been detected in toxic and nontoxic (E-like) strains of C. botulinum type E (Scott and Duncan, 1978) but no relationship of these plasmids to boticinogenicity, antibiotic resistance, or toxicity has been reported. Currently, there is no known function for the plasmids of C. botulinum type A or C. sporogenes.

Differentiation of C. botulinum type A and C. sporogenes by plasmid profile was successful for the test strains containing plasmid DNA. C. botulinum type A plasmids were of only two sizes while C. sporogenes plasmids varied in size and were generally larger than those detected in the C. botulinum test strains. None of the plasmids seen from C. sporogenes were of the same molecular weight as those from C. botulinum type A (Table 1). Ideally, the screening of more C. botulinum type A and C. sporogenes strains by this method is necessary to make a stronger conclusion about the role of plasmids in these organisms.

D. Plasmid Molecular Weight Determination

A representative standard curve constructed from the gel shown in Figure 13 is shown in Figure 14. E. coli V517 plasmids of 35.8, 4.8, 3.7, and 3.4 Mdal were used as markers because RNA masked the smallest (2.0, 1.8, and 1.4 Mdal) plasmids in the agarose gels. These markers also covered the size range of the unknown plasmids and consistently

Figure 14. Standard curve of logarithm molecular weight (Mdal) versus logarithm migration distance (cm) of plasmids from reference strains *E. coli* V517 and *E. stewartii* SW_2 in 0.7% agarose gels.


E. stewartii SW2
E. coli V517
log MOLECULAR WEIGHT (Md)
1.5
1.3
1.1
0.9
0.7
0.5
0.3
.4
.5
.6
.7
.8
.9
1.0
log DISTANCE (cm)

gave accurate migration distances. Only five (34.5, 33.0, 29.5, 8.8, and 2.8 Mdal) of twelve E. stewartii SW_2 plasmids were used as standards for the same reasons as above. The 23.2 and 16.9 Mdal plasmids of E. stewartii were eliminated from the standards because of inconsistencies in their mobility in agarose gels (Dr. D. Coplin, 1982, private communication).

Extrapolated molecular weight estimates from all standard curves for C. botulinum type A and C. sporogenes plasmids were averaged and standard deviations were calculated (Table 1).

For those strains of C. botulinum type A containing plasmids, there were always two plasmids, one 15 to 15.6 Mdal and the other 6.2 Mdal. The C. sporogenes strains showed more diversity in plasmid molecular weight. C. sporogenes strain 2669 had two plasmids (24.3 and 9.9 Mdal) and strains 4131C and 3679 had one plasmid each (25.8 and 40.6 Mdal respectively), while no plasmids were detected in the other four strains screened.

No relationship between plasmid profile and the results from the biochemical tests, antibiotic resistance, or polyacrylamide gel electrophoresis was found in this species. For example, C. sporogenes test strains had diverse plasmid profiles and similar phenotypic test patterns.

With a rapid plasmid screening procedure, this type of data may be helpful in differentiating C. botulinum type A from the closely related C. sporogenes without toxin testing. The extreme genetic relatedness between these species, as discussed by Lee and Riemann (1970a), Lee and Riemann (1970b), and Wu et al. (1972), may differ in as little genetic material as a plasmid(s) or transposon(s).

Table 1. Molecular weight estimation of plasmids from C. botulinum type A and C. sporogenes from mobility in 0.7% agarose gels.

Organism	Strain # & Type	[a] Molecular Weight ± Standard Deviation	Figures
Clostridium botulinum	62A	15.0 ± 1.3	5,8,12,13
		6.2 ± 0.24	
	52A	15.0 ± 1.3	8,12,13
		6.2 ± 0.16	
	36A	15.6 ± 0.99	7,11,13
		6.3 ± 0.23	
	33A	15.6 ± 0.99	7,9,10,13
		6.2 ± 0.02	
	10755A	NP	9
Clostridium sporogenes	2669	24.3 ± 1.8	6,12,13
		9.9 ± 0.41	
	4131C	25.8 ± 2.4	6,12,13
	3679	40.6 ± 3.7	10,12,13
	2022	NP	6
	77	NP	6
	6054D	NP	9,11
	1963	NP	11

NP = No Plasmids Detected

a = E. coli V517 and E. stewartii SW_2 plasmids used as molecular weight markers (Figure 14). At least 3 measurements were taken for each test strain plasmid.

One of the strains examined in this study, C. botulinum 62A, had previously been screened for plasmids by Scott and Duncan (1978). Strain 62A was the only type A C. botulinum included in their study. Their procedure included a cesium chloride-ethidium bromide gradient to isolate C. botulinum plasmids and electron micrographs to measure contour lengths, which were converted to molecular weights. Two plasmids were detected in C. botulinum 62A by Scott and Duncan (1978) and they were reported as being 6.9 ± 0.2 Mdal (85 measurements) and 18.9 Mdal (1 measurement). The small differences in reported molecular weights between this study and those published by Scott and Duncan (1978) may have been due to procedural differences, but there is a general agreement as to the number and size of the plasmids in this organism.

Plasmid curing and more extensive genetic studies are needed to determine the characteristics of these plasmids and the genetic control of toxin production. Strict precautions must be taken if cloning experiments are to be tried because of the nature of botulinum toxin.

SUMMARY AND CONCLUSIONS

A rapid plasmid screening procedure was developed for C. botulinum type A and C. sporogenes to determine the plasmid profile of several strains of each bacterium. The relationship of plasmids to toxin production in C. botulinum type A was examined, and an evaluation as to whether these closely related species can be differentiated based on plasmid profile is discussed.

Phenotypic characteristic screening, toxin typing, and polyacrylamide gel electrophoresis of soluble cellular proteins were performed on each strain to confirm identity.

The plasmid isolation procedure was a modified cleared lysate technique. Spheroplasts were formed with a bacteriolytic enzyme and lysis was completed by the addition of sodium dodecyl sulfate under alkaline conditions. Salt precipitation and phenol extractions removed the bulk of the chromosomal DNA and protein. Ethanol precipitates of test strain DNA and plasmid molecular weight markers were analyzed in 0.7% agarose gels to estimate the sizes of the test strain plasmids.

The effect of culture age on the plasmid isolation procedure was examined since cell density and the ability to lyse the cells varied with the stage of growth. Young cultures lysed well, but contained less DNA due to a lower cell density. Old cultures did not lyse well and plasmid detection was often decreased. Excess chromosomal DNA, which masked the plasmids in the agarose gels, also caused problems when stationary phase cells were used. Middle to late log cultures lysed efficiently and contained sufficient cells for optimal plasmid detection.

Four out of five toxic C. botulinum type A strains and three out of seven C. sporogenes strains contained extrachromosomal DNA. Plasmid molecular weights were extrapolated from standard curves produced from the migration of standards in agarose gels. All plasmid-containing C. botulinum type A strains showed uniformity in the presence of two plasmids of around 6 and 15 Mdal. Two of the plasmid-containing C. sporogenes strains had one plasmid. The C. sporogenes strain 4131 plasmid was estimated to be 25.8 ± 2.4 Mdal and C. sporogenes 3679 plasmid was 40.6 ± 3.7 Mdal. C. sporogenes strain 2669 had two plasmids which corresponded to 25.8 ± 2.4 and 9.9 ± .41 Mdal.

Although no plasmids were detected in one of the five C. botulinum type A strains, plasmids may still be responsible for toxin production. Integration of the plasmids into the chromosome could explain why no extrachromosomal DNA was detected in the one toxic strain. Curing experiments, leading to the elimination of one or both of the C. botulinum type A plasmids, would provide more information about the role of plasmids in toxin production.

The ability to differentiate the culturally and serologically identical C. botulinum type A and C. sporogenes by plasmid profile was successful for the plasmid containing strains. C. botulinum type A strains showed two constant plasmid bands in the agarose gels, while C. sporogenes showed no consistency. C. sporogenes test strains had no plasmid sizes in common with the plasmid-containing C. botulinum type A strains and in general the plasmids of C. sporogenes were of a greater molecular weight than those detected in C. botulinum type A.

The functions of the plasmids detected in C. botulinum type A and C. sporogenes are yet unknown.

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PLASMIDS IN *CLOSTRIDIUM BOTULINUM* TYPE A

AND *CLOSTRIDIUM SPOROGENES*

by

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(ABSTRACT)

A procedure to rapidly screen *Clostridium botulinum* type A and *Clostridium sporogenes* for plasmids was developed. Plasmid profiles of five *C. botulinum* type A strains and seven *C. sporogenes* strains were determined and a possible relationship of plasmids to toxin production was examined. The differentiation of these organisms by plasmid fingerprinting was also studied. The plasmid isolation procedure was a modified cleared lysate technique, including lysis under alkaline conditions. Samples were subject to agarose gel electrophoresis to detect plasmid DNA. Culture age affected plasmid detection due to changes in the cell density and lysing efficiency. Middle to late log cultures were used throughout the study because they provided optimum plasmid detection. Four out of five *C. botulinum* type A strains and three out of seven *C. sporogenes* strains contained extrachromosomal DNA. For those *C. botulinum* type A strains which contained plasmids, there were always two, one 15 to 15.6 Mdal and the other 6.2 Mdal. *C. sporogenes* showed less consistency in plasmid size and number and their plasmids were generally of a greater molecular weight than those in *C. botulinum* type A. One *C. sporogenes* strain contained two plasmids and two strains contained one plasmid. Toxin production may be plasmid-mediated in the plasmid-containing strains, but there was no apparent general relationship, because one of the toxic strains did not show the presence of plasmids.

Plasmid screening may be useful in the differentiation of these closely related organisms without toxin testing.