APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1991, p. 1018–1025
0099-2240/91/041018-08$02.00/0

Vol. 57, No. 4

Selection of Colony, Plasmid, and Virulence Variants of *Staphylococcus epidermidis* NRC853 during Growth in Continuous Cultures Exposed to Erythromycin

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Received 19 November 1990/Accepted 5 February 1991

A continuous-culture system was developed to study changes in the structure of *Staphylococcus epidermidis* populations exposed to subminimum inhibitory concentrations of erythromycin. Continuous-culture experiments were carried out in a dextrose-free, tryptic soy broth medium supplemented with lactic acid and sodium lactate (MTSB-D). The multiresistant (penicillin-, tetracycline-, and erythromycin-resistant) *S. epidermidis* strain NRC853 was subjected to a series of experiments: (i) growth individually in continuous culture in the absence and presence of erythromycin and (ii) growth in mixed culture with the erythromycin-susceptible *S. epidermidis* strain NRC852 in the absence and presence of erythromycin. Strain NRC853 produced colony morphology variants during continuous culture in the presence of 0.05 and 0.1 μg of erythromycin per ml. Variants (A, B, and C) were different from their wild-type parent on the basis of colony size, sector pattern, and/or the ability to transmit light. A variants rapidly lost a 2.7-MDa tetracycline resistance plasmid. B and C variants formed an *ermC* plasmid multimer series from unit size to a 16-mer and exhibited an approximately twofold increase in erythromycin MIC over that of the wild-type parent. They slowly lost the tetracycline resistance plasmid. The small-colony B variant demonstrated an increased virulence in the neonatal mouse weight gain test and an increase in fibronectin binding compared with the wild-type parent. The presence of a competing strain drastically increased the frequency of all variants.

Staphylococcus species constitute a major component of the mammalian cutaneous microflora (7, 8, 13). Comprehensive investigations have been made of the natural populations of staphylococci, especially of those living on humans and other primates and domestic animals (4, 9–11, 14). However, very few studies examining staphylococcal community structure in laboratory systems have been conducted. Marsh and Selwyn (20) examined the antagonistic effects of a strain of a coagulase-negative *Staphylococcus* sp. against an indicator strain of a *Micrococcus* sp. during continuous culture in a chemostat. Their work demonstrated the production of an antibiotic by the staphylococcus when the growth medium supported at least 10^7 CFU/ml, an observation supported by results of previous liquid batch cultures and growth on agar media (19). These investigators concluded that the continuous culture techniques offered many obvious advantages over batch cultures but fell somewhat short of establishing natural conditions by not providing a solid surface for the growth of organisms and the retention of microbial products.

In the following study, we have investigated the responses of a multiple antibiotic-resistant strain of the coagulase-negative species *Staphylococcus epidermidis* in pure and mixed continuous cultures to subminimum inhibitory concentrations of penicillin G, tetracycline, or erythromycin. Low levels of these antibiotics may be present in the natural cutaneous habitats of this species during patient therapy (3). *S. epidermidis* is an opportunistic pathogen that has been implicated in a variety of infections, most of which are the result of the use of prosthetic and indwelling devices in human medicine and immunosuppressive therapy (5, 24). This species is the most prevalent and persistent *Staphylococcus* species on human skin and produces large multiresistant populations during the course of single-antibiotic therapy (8, 9).

Our initial objective was to develop a continuous culture system capable of growing single and mixed cultures of *S. epidermidis* for several days and then to use that system to observe changes in strain and community structure during antibiotic pressure, in an attempt to define adaptive processes. Preliminary results of colony variation in continuous cultures of multiresistant *S. epidermidis* have been presented previously (3a).

MATERIALS AND METHODS

Bacterial strains and source. *S. epidermidis* NRC853 was examined in all continuous cultures. *S. epidermidis* NRC853 and NRC852 were tested in competition experiments.

NRC853 was multiply resistant to erythromycin (MIC, ≥1,600 μg/ml), penicillin G (MIC, 50 μg/ml), and tetracycline (MIC, 50 μg/ml) and was isolated from the forehead of a 3-year-old girl receiving amoxicillin for otitis media. This strain was the predominant one present on the forehead prior to and during amoxicillin therapy. Two colony morphotypes were present in natural populations of NRC853. One morphotype produced large (7.0- to 7.1-mm diameter), raised, gray (translucent) colonies, and the other produced medium-size (4.5- to 5.8-mm diameter), gray-white colonies with a slightly depressed center. Some of the large gray colonies contained more slowly growing gray-white sectors. Cells isolated from these sectors produced colonies similar to the medium-size, gray-white colonies mentioned above. All of the medium-size, gray-white colonies but not all of the large gray colonies were multiply resistant to erythromycin, penicillin, and tetracycline. Some of the large gray colonies were resistant to penicillin and tetracycline or penicillin and

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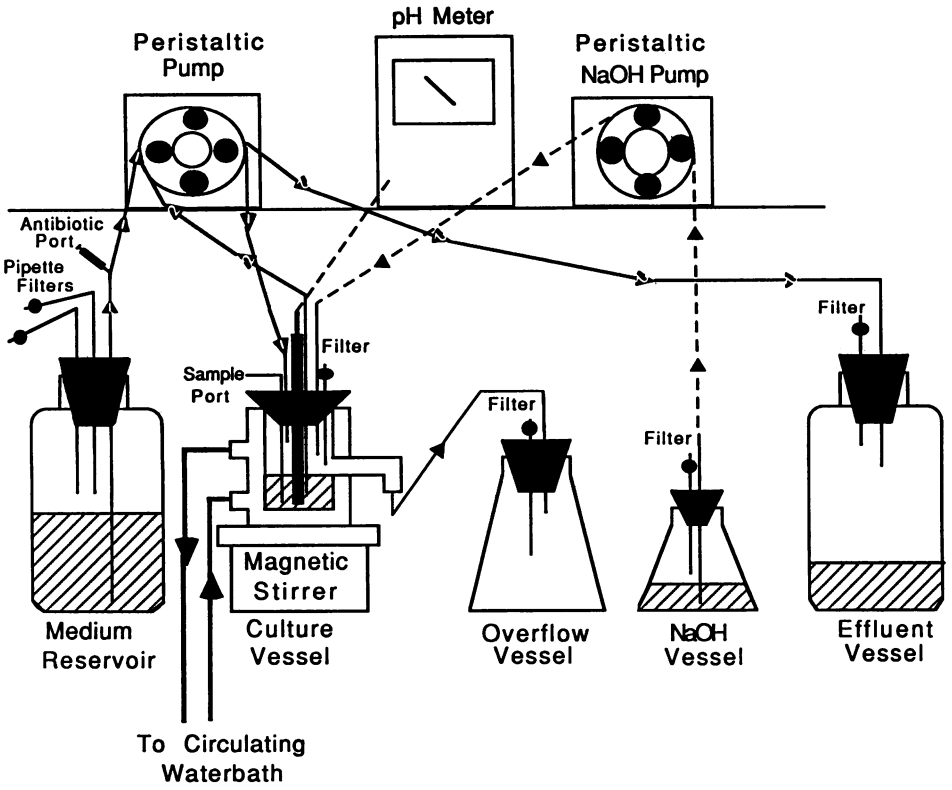

FIG. 1. Continuous culture apparatus for the growth of *S. epidermidis*. The path of the medium feed line (arrows marked 1) and effluent line (arrows marked 2) are indicated by a solid line, and the path of the NaOH line is indicated by a dashed line.

erythromycin. For the present study, inocula were prepared from only the medium-size, gray-white colony morphotype. NRC852 was resistant to penicillin G (MIC, 200 μg/ml) and susceptible to erythromycin (MIC, 0.5 μg/ml) and tetracycline (MIC, 3.1 μg/ml). It was isolated from the same forehead community as NRC853 and initially at the same time. Following 10 days of therapy with amoxicillin, NRC852 was displaced on the forehead by the multiresistant strain NRC853.

Bacterial cultures were examined for species and strain identification by the methods of Kloos and coworkers (8–15). Cultures were maintained on P agar (28) at 4°C for up to 2 months and preserved by desiccation on steatite fish spine insulator beads at 4°C. These storage conditions did not alter the antibiograms or plasmid compositions of the above strains.

S. epidermidis NRC8540(pWE204) was used as the source of the *ermC* gene probe. *Staphylococcus aureus* RN2442 (pE194) and *S. epidermidis* NRC8540 were used as references for the *ermC* gene in hybridization experiments. *S. aureus* RN11(pI258) was a reference for the *ermB* gene, and RN4931(pEM9714) was a reference for the *ermA* gene. The *S. aureus* reference strains were kindly supplied by Ellen Murphy (The Public Health Research Institute, New York, N.Y.).

Medium composition and preparation. The medium used for continuous culture experiments was tryptic soy broth without dextrose (TSB-D) (Difco) supplemented with lactic acid (85%, certified by the American Chemical Society) (1.48 ml/liter) and sodium lactate (syrup, about 60%) (2.08 ml/liter) (MTSB-D). Glucose (dextrose) was omitted from the medium to reduce the production of slime, which if allowed to accumulate will aggregate cells in culture, adhere cells to the wall of the culture vessel, and clog the tubing of the continuous-culture system. The addition of a lactic acid-sodium lactate buffer to the medium provided a nonlimiting energy source similar to that encountered by staphylococci in their natural cutaneous habitat (28) and did not promote extensive slime production. The medium was prepared as follows. A solution containing 5 to 8 liters of distilled water, TSB-D, lactic acid, and sodium lactate was mixed in a 9.5- or 19-liter glass (Pyrex) carboy (medium reservoir) that was connected by tubing to the culture vessel. The continuous-culture apparatus is illustrated in Fig. 1. A peristaltic pump was used to maintain a dilution (*D*) rate of 0.3 or 0.4/h, rates that were chosen to remove cells and limit the population size to maximum cutaneous densities (10) without limiting nutrients. The pump was also used, acting in the reverse direction, to add an antibiotic solution to the medium. The entire continuous-culture system (excluding the peristaltic pumps and pH controller-meter) was autoclaved as a unit for 45 min at 121°C and then cooled. The antibiotic solution was added when the medium was cooled to 35°C.

P agar was used to prepare fresh cultures for all experiments, screen continuous-culture populations for cell density and colony morphology, and provide the base medium for determining antibiograms and for antibiotic (erythromycin, penicillin G, or tetracycline)-containing agars used in replica plating. Brain heart infusion agar (Difco) supplemented with 1% glycine was used to propagate cultures for plasmid isolation. The MIC of antibiotics was determined in Mueller-Hinton broth.

Continuous-culture system. The culture vessel (Fig. 1) contained a water jacket that was connected to a circulating

water bath maintained at 35°C. The working volume of the culture vessel was 50 ml and was maintained by overflow through a sidearm in the vessel. The culture vessel stopper was fitted with four glass tubes of 4-mm outside diameter, one glass tube of 6-mm outside diameter, and a pH electrode (model 465-35-90; Ingold Electrodes, Inc., Andover, Mass.). One of the four glass tubes was connected to the medium feed line, one was connected to the effluent line, one was connected to tubing leading to the sodium hydroxide (NaOH) vessel, and another was used as a sample port. The sample port tube was extended to the midpoint of the culture volume. The larger glass tube was connected to a bacterial air vent. The culture was mixed with the aid of a Corning stirrer driving a Teflon-coated, magnetic stir bar (1.5 by 8 mm) inside the culture vessel. pH was maintained at 6.5 by a Cole-Parmer pH controller (model 5997-20) receiving a signal from the pH electrode (see above) and controlling the power to a peristaltic pump connected to the NaOH vessel.

Continuous cultures were started by turning on the feed peristaltic pump, filling the culture vessel with medium to the overflow, and then stabilizing the temperature, pH, and dilution rate (requiring approximately 5 h). The culture vessel medium was inoculated with a 0.5-ml aliquot of an *S. epidermidis* culture grown for 4 h in TSB-D (5 ml in a Klett colorimeter tube) at 35°C and with shaking (200 rpm) and adjusted to an optical density of 0.36 with sterile TSB-D. The broth tubes were inoculated to an initial optical density of 0.1 ± 0.02 with cells grown overnight on P agar at 35°C.

Sampling procedures. Continuous cultures were maintained for a period of 3 to 5 days. They were sampled at the beginning (time zero) and then daily for the duration of the experiment. Each 0.5-ml sample was diluted in 4.5 ml of sterile saline (0.85% NaCl). Serial $\log_{10}$ (10-fold) dilutions of this suspension to $1/1 \times 10^6$ were prepared in 4.5 ml of sterile saline. Aliquots (0.1 ml) of the $1/1 \times 10^2$ to $1/1 \times 10^6$ dilutions were spread on triplicate P-agar plates. Plates were incubated at 35°C for 3 days and maintained at room temperature for an additional 2 days. At 2 days of incubation, colonies on one of the triplicate plates prepared from each dilution were replica plated onto a series of three P-agar plates containing erythromycin (25 μg/ml), tetracycline (20 μg/ml), and penicillin G (1 μg/ml), respectively. The viable count, purity of the culture, antibiotic resistance pattern, and colony morphology were determined from plates showing approximately 50 to 500 colonies.

Antibiotic resistance profile. Following the replication of colonies onto antibiotic-containing agars (see above), plates were incubated at 35°C for 2 days and then examined for the presence of colonies that showed an antibiotic susceptibility different from that of the parent strain. The suspected variant colonies were isolated and examined for their antibiotic resistance profiles (antibiograms), by using standard agar disk diffusion (23) and broth macrodilution (22) methods and plasmid composition.

Analysis of colony morphology variants. Colonies that were different from the parent strain on the basis of size, light transmission, color, profile, edge, surface, and/or consistency were designated as colony morphology variants. Colonial properties were best differentiated following incubation of colonies for 3 days at 35°C and 2 days at room temperature on P agar. Variant and parent colonies were isolated, subcultured, and reexamined for colonial morphology on point-inoculated P-agar plates (11). Several representative colonies of each morphotype were tested for antibiogram (above) and plasmid composition and were further subjected to a battery of biochemical and physiological tests used for species and strain identification (see above) involving carbohydrate reactions, enzyme assays, hemolysin production, and anaerobic growth in thioglycolate, to determine specific parentage. Each colony morphotype of strain NRC853 was tested for pathogenicity in the neonatal mouse weight gain test (6) (courtesy of B. A. Gunn, Converse, Tex.); particle agglutination assays with laminin, collagen type IV, and gelatin (21); and binding of ^{125}I-labeled fibronectin (Fn) (27) (courtesy of T. Wadström, University of Lund, Lund, Sweden).

Preparation of plasmid and chromosomal DNA. Cultures were prepared for plasmid isolation by first spreading an overnight P-agar culture onto the surface of a brain-heart infusion–1% glycine agar plate. The surface area inoculated per culture was approximately 15 by 30 mm. Inoculated plates were incubated at 35°C for 16 to 18 h. Cells were harvested from the agar surface with an inoculating loop and suspended in 1.5 ml of ice-cold (100 mM) NaCl–25 mM Tris-Cl–10 mM EDTA (pH 7.5) buffer in an Eppendorf tube. The cell suspension was vortexed to reduce the size of cell aggregates and produce as homogeneous a suspension as practically possible. The cell suspension was centrifuged for 1 min in an Eppendorf centrifuge, and the supernatant fluid was decanted. The cell pellet was resuspended in 150 μl of an ice-cold solution of lysing buffer (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA, pH 7.5) containing lysostaphin (1 to 2 μg/ml) and was vortexed immediately. The lysing cell suspension was stored in an ice bath for 10 min and then at room temperature for 10 to 20 min until partial lysis (the start of a translucent appearance and increase in viscosity) was noted. Once partial lysis was noted, the suspension was frozen at −20°C until the next working day and then thawed at 35°C and stored at this temperature until lysis was complete or close to completion, usually requiring 15 to 30 min. The lysed cell suspension was treated for plasmid isolation by a modification of the Birnboim and Doly alkaline denaturation procedure, as described by Maniatis et al. (18). Chromosomal DNA was isolated for several hybridization experiments using *erm* gene probes. The isolation procedure was similar to that used for plasmids except that the alkaline denaturation step was omitted and 10 M ammonium acetate was used in place of potassium acetate. The final volume of the purified plasmid or chromosomal DNA preparation was 20 μl.

Agarose gel electrophoresis. From the purified plasmid preparation (see above) duplicate 2-μl aliquots were used for vertical agarose gel electrophoresis to obtain a plasmid profile and 16 μl was used for low-melting-temperature (LMT) agarose gel electrophoresis to isolate specific plasmids from a profile (18). A supercoiled DNA ladder (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md.) and a purified plasmid preparation from *Escherichia coli* V517 (17) were used as sources of size reference plasmid molecules.

Vertical agarose gels (12 cm by 14 cm by 3 mm, 20 wells) were cast and run in a dual vertical slab gel electrophoresis cell (model 220; Bio-Rad Laboratories, Richmond, Calif.). The gel contained 0.8 or 1.2% agarose in Tris-borate buffer (18) with 1% sodium dodecyl sulfate. Tris-borate buffer was also used for electrophoresis. Plasmid profile gels were subjected to 50 V for 30 min before loading (prerun) and then run at 50 V for 1 h and at 70 V for the remainder of the run. Gels were stained by immersion in distilled water containing ethidium bromide (1.5 μg/ml) for 10 min, followed by destaining in distilled water for 10 min. Gels were illuminated with a Chromato-Vue transilluminator (model TM36; Ultra-

Violet Products, Inc., San Gabriel, Calif.) and photographed with a Polaroid MP-4 Land Camera System (Polaroid Corp., Cambridge, Mass.) using a Kodak #9 yellow Wratten filter (Eastman Kodak Co., Rochester, N.Y.) and Polaroid type 655 black and white film.

LMT agarose gels (15 cm by 15 cm by 4 mm, 10 wells) were cast and run in a Wide Mini-Sub Cell (Model 1704343; Bio-Rad). The gel contained 1.0 or 1.2% Sea Plaque agarose (FMC Bio Products, Rockland, Maine) in Tris-borate buffer with ethidium bromide (0.5 μg/ml). Tris-borate buffer was used for electrophoresis and covered the horizontal gel by a depth of 2 mm. Gels were prerun at 15 V for 30 min and then run at 15 V for 1 h, 25 V for 1.5 h, 40 V for 1 h, and 60 V for 2 h. Chromosomal DNA preparations were also subjected to LMT agarose gel electrophoresis and processed in a way similar to that for plasmids, except that the electrophoresis buffer covered the gel by a depth of 4 mm to facilitate loading of the viscous sample into the well and the run was concluded with the 40-V portion extended for 4 h. DNA was recovered from the LMT agarose gel by the procedures of Weislander (30).

Restriction endonuclease analysis. Plasmids were digested by the restriction enzymes *Cfo*I, *Dra*I, *Eco*RI, *Hae*II, *Hin*cII, *Hin*fI, *Msp*I, and *Rsa*I, and chromosomal DNA was digested by the restriction enzymes *Cfo*I, *Eco*RI, *Hin*fI, and *Msp*I (Bethesda Research Laboratories Life Technologies, Inc.) according to the recommended procedures of the manufacturer. Preparative plasmid digests contained 0.8 to 3.0 μg of DNA and chromosome digests contained 15 to 30 μg of DNA in a final reaction volume of 30 μl. Restriction fragments were separated by vertical agarose gel electrophoresis (see above) on gels containing 1.4 or 2.2% agarose. Linear DNA molecular size markers included an *Msp*I digest of pBR322 (New England BioLabs, Beverly, Mass.) and a 123-bp ladder (Bethesda Research Laboratories Life Technologies, Inc.).

Preparation of *erm* gene probes. The *ermC* probe was a 644-bp *Hin*fI-*Hin*cII fragment from pWE204. It contained 47 bp of the leader region (including the Shine-Dalgarno sequence SD-2) and 597 bp of the 23S rRNA methyltransferase gene (16). pWE204 was isolated from the macrolide-lincosamide-streptogramin B-inducible *S. epidermidis* strain NRC8540 by alkaline denaturation procedures and preparative LMT agarose gel electrophoresis and extraction (see above). Purified pWE204 DNA was codigested with the restriction enzymes *Hin*fI and *Hin*cII, and the restriction fragments (1,063, 644, 309, 230, and 216 bp) were separated and purified by using LMT agarose gel electrophoresis and extraction procedures (see above). The 644-bp *Hin*fI-*Hin*cII fragment preparations contained 3.5 to 4.0 μg of DNA in a final volume of 10 μl. This preparation was mixed with an equal volume of photoactivatable biotin (1 μg/μl; Research Organics, Inc., Cleveland, Ohio), placed in an ice bath, and irradiated with a Sylvania sunlamp (275 W, model RSM/H; GTE Products Corp., Manchester, N.H.) at a distance of 10 cm for 15 min. The photobiotin-labeled DNA probe was purified by using the procedures recommended by Clontech (Palo Alto, Calif.).

The *ermA* probe was prepared from the phage M13mp11 containing a 417-bp *Mbo*I fragment internal to the *ermA* of Tn*554* (nucleotides 4706 to 5123) inserted in the *Bam*HI site of M13mp11 (clone M-977 having the same sequence as *ermA* mRNA). Phage were propagated on *E. coli* JM105 and were kindly supplied by E. Murphy. M-977 DNA was labeled with photoactivable biotin (see above).

DNA transfer and hybridization. Agarose gels were depurinated in 0.25 M HCl twice for 15 min each time. They were denatured in a solution containing 1.5 M NaCl and 0.5 M NaOH for 1 h and then neutralized in neutralizing buffer containing 10 mM Tris (pH 7.8), 5 mM sodium acetate, and 0.5 mM EDTA twice for 1 h each time. The transfer of DNA from agarose gels was performed by using a Bio-Rad Trans-Blot cell and model 250/2.5 power supply (Bio-Rad Laboratories) by procedures recommended by the manufacturer. DNA was transferred to Hybond N nylon membranes (Amersham Corp., Arlington Heights, Ill.). Prehybridization treatment and hybridization using the above *erm* gene probes were done according to the procedures described by Wahl et al. (29) but modified for use with *Staphylococcus* DNA (T_m = 82 to 87°C). Prehybridization and hybridization mixtures contained 50 and 45% deionized formamide, respectively, in addition to standard ingredients. Dextran sulfate was omitted from the hybridization mixture. Prehybridization and hybridization were performed at 42°C. The colorimetric detection of photobiotinylated DNA in hybridizations was made by the BluGENE Nonradioactive Nucleic Acid Detection System (Bethesda Research Laboratories Life Technologies, Inc.).

RESULTS

Appearance of colony morphology variants in continuous cultures. *S. epidermidis* NRC853 was grown alone and with strain NRC852 in MTSB-D without (control) and with a subminimum inhibitory concentration of penicillin G (0.8 μg/ml), tetracycline (0.05 μg/ml), or erythromycin (0.05 μg/ml). Colony morphology variants were selected during the course of continuous culture in the presence of erythromycin but not in the presence of penicillin G or tetracycline. For the remainder of the study, only the effects of erythromycin on variation were examined. Colony morphology was examined at 0, 0.005, 0.05, 0.1, 0.5, and 5 μg of erythromycin per ml. The results (Table 1) show that the frequency of the C variant increased with 0.05 or 0.1 μg of erythromycin per ml and increased even more with erythromycin when strain NRC852 was present. In the presence of erythromycin, NRC852 was eventually eliminated from cultures in which the initial inoculum contained nearly equal numbers of competing strain CFU. In the absence of erythromycin, NRC852 eventually displaced the competing strain. Colony morphology variants were not observed for strain NRC852 in any of the experiments.

NRC853 produced three colony morphology variants (Fig. 2). At time zero, the culture contained 5×10^7 to 10×10^7 CFU/ml. At D = 0.3/h and in the presence of 0.05 μg of erythromycin per ml, the culture reached a stable density of 5×10^8 to 6×10^8 CFU/ml by the second day. At D = 0.4/h, the culture density declined to 3×10^6 to 6×10^6 CFU/ml between the first and second day and remained at this level. The frequency and time of appearance of colony morphology variants was only slightly different at the two dilution rates. The A variant appeared only when erythromycin and the competing strain NRC852 were present in the culture. This variant appears to be the same as the large gray morphotype isolated from the forehead of the patient (above). The B variant produced a very small (2- to 3.5-mm diameter), gray-white colony with a dark gray center, and it sometimes contained white sectors. Sectored colonies were more prevalent in cultures in which D = 0.3/h. The C variant produced a small to medium-size (3.5- to 6.0-mm diameter), gray-, gray-white-, and white-sectored colony.

Antibiotic resistance and plasmid composition. NRC853

TABLE 1. Frequency of colony morphology variants of *S. epidermidis* NRC853 grown in continuous cultures with and without erythromycin

Experiment and time (days)	% CFU[a]				
	NRC852[b]	NRC853[c]			
		WT	A	B	C
Without erythromycin					
D = 0.3/h					
0		100	0	0	0
1		100	0	0	0
2		99.9	0	0	<0.1
3		99.9	0	0	<0.1
D = 0.4/h					
0		99.9	0	0	<0.1
1		99.9	0	0	0.1
2		99.7	0	0	0.3
3		99.1	0	0.3	0.6
With NRC852 (1:1), D = 0.3/h					
0	42.3	57.7	0	0	0
1	91.0	9.0	0	0	0
2	92.9	7.1	0	0	0
3	92.8	7.2	0	0	<0.1
With erythromycin (0.05 μg/ml)					
D = 0.3/h					
0		99.9	0	0	<0.1
1		99.9	0	0	<0.1
2		97.3	0	0	2.7
3		81.1	0	8.4	10.5
D = 0.4/h					
0		99.9	0	0	0.1
1		96.2	0	0	3.8
2		97.3	0	0	2.7
3		97.2	0	0	2.8
With NRC852 (1:1), D = 0.3/h					
0	41.5	58.5	0	0	0
1	57.4	42.6	0	0	0
2	7.9	15.8	19.7	47.4	9.2
3	6.9	6.9	5.7	57.5	23.0
With NRC852 (1:1), D = 0.4/h					
0	56.5	43.5	0	0	0
1	28.1	70.3	0	1.6	0
2	10.0	73.6	5.0	4.7	6.7
3	0	73.3	2.0	12.7	12.0
With NRC852 (2:1), D = 0.3/h					
0	70.6	29.4	0	0	0
1	6.3	17.5	22.2	50.8	3.2
2	16.2	6.3	32.5	31.2	13.8
3	7.2	7.3	8.7	55.1	21.7
With erythromycin (0.1 μg/ml) and NRC852 (1:1), D = 0.3/h					
0	43.7	56.3	0	0	0
1	7.7	92.3	0	0	0
2	2.1	57.4	21.3	6.4	12.8
3	0.7	9.4	4.3	18.1	67.5
With erythromycin (0.5 μg/ml) and NRC852 (1:1), D = 0.3/h					
0	58.9	41.1	0	0	0
1	12.7	87.3	0	0	0
2	3.7	96.3	0	0	0
3	3.3	89.0	0	7.7	0

[a] Colony morphology variants were not observed in competition experiments with 0.005 or 5.0 μg of erythromycin per ml. Experiments at D = 0.3/h were repeated two to four times, and data shown are the mean percent CFU. The frequency of variant type and time of appearance were not noticeably different between replicate experiments. Experiments at D = 0.4/h were performed once.

[b] NRC852 is susceptible to erythromycin (MIC, 0.5 μg/ml) and is a strain of *S. epidermidis* used in competition experiments.

[c] NRC853 is resistant to erythromycin (inducible *ermC*) (MIC, ≥1,600 μg/ml). WT, Wild-type parent; A, large, translucent-gray colony variant; B, small, gray-white-, and white-sectored colony variant; C, gray-, gray-white-, and white-sectored colony variant.

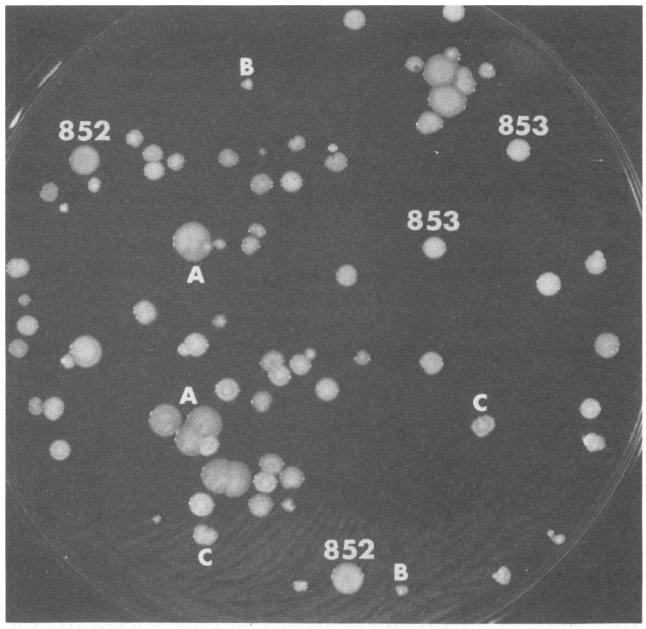

FIG. 2. Colonies of *S. epidermidis* NRC852 and NRC853 from cells grown in continuous culture for 3 days in the presence of a subminimum inhibitory concentration of erythromycin. Colony designations: 852, NRC852 wild type; 853, NRC853 wild-type parent; A, NRC853 A variant; B, NRC853 B variant; C, NRC853 C variant. Colonies were grown for 3 days at 35°C and 2 days at room temperature on P agar.

colony morphology variants demonstrated antibiotic resistance instability, and this was associated with a loss of specific plasmids. The A variant rapidly lost tetracycline resistance and the associated 2.7-MDa tetracycline-resistance plasmid during continuous culture. On the second day of culture (in the presence of erythromycin), approximately 60% of A-variant CFU were susceptible to tetracycline, and by the fourth day, >90% were susceptible to this antibiotic. The B and C variants also lost tetracycline resistance when grown in the presence of erythromycin. On the second day, approximately 12% of B-variant CFU were susceptible to tetracycline, and by the fourth day, 38% were susceptible. Several of the B-variant tetracycline-resistant colonies demonstrated a reduced tetracycline resistance plasmid copy number (less than 25% of the number of the parent), but this change was not associated with a noticeable change in MIC. The rate of loss of antibiotic resistance was more difficult to determine with the C variant because of frequent sectoring in developing colonies. Sectors that were different in their ability to transmit light (gray, gray-white, or white sectors) and were well defined were each tested for antibiotic resistance profile and plasmid composition. In some colonies, the type of sector was associated with a specific resistance profile. Both B and C variants demonstrated an approximately twofold increase in erythromycin MIC compared with that of the wild-type parent. The plasmid profile and results of hybridization with an *ermC* probe indicated that these variants formed an *ermC* plasmid multimer series (Fig. 3). The series proceeded from a high-copy-number, unit-size plasmid of 1.62 MDa to a 16-mer of 25.9 MDa. The even-numbered multimers 4-mer to 16-mer were present in higher copy number than the odd-numbered multimers 5-mer to 15-mer. The wild-type parent and A variant produced low

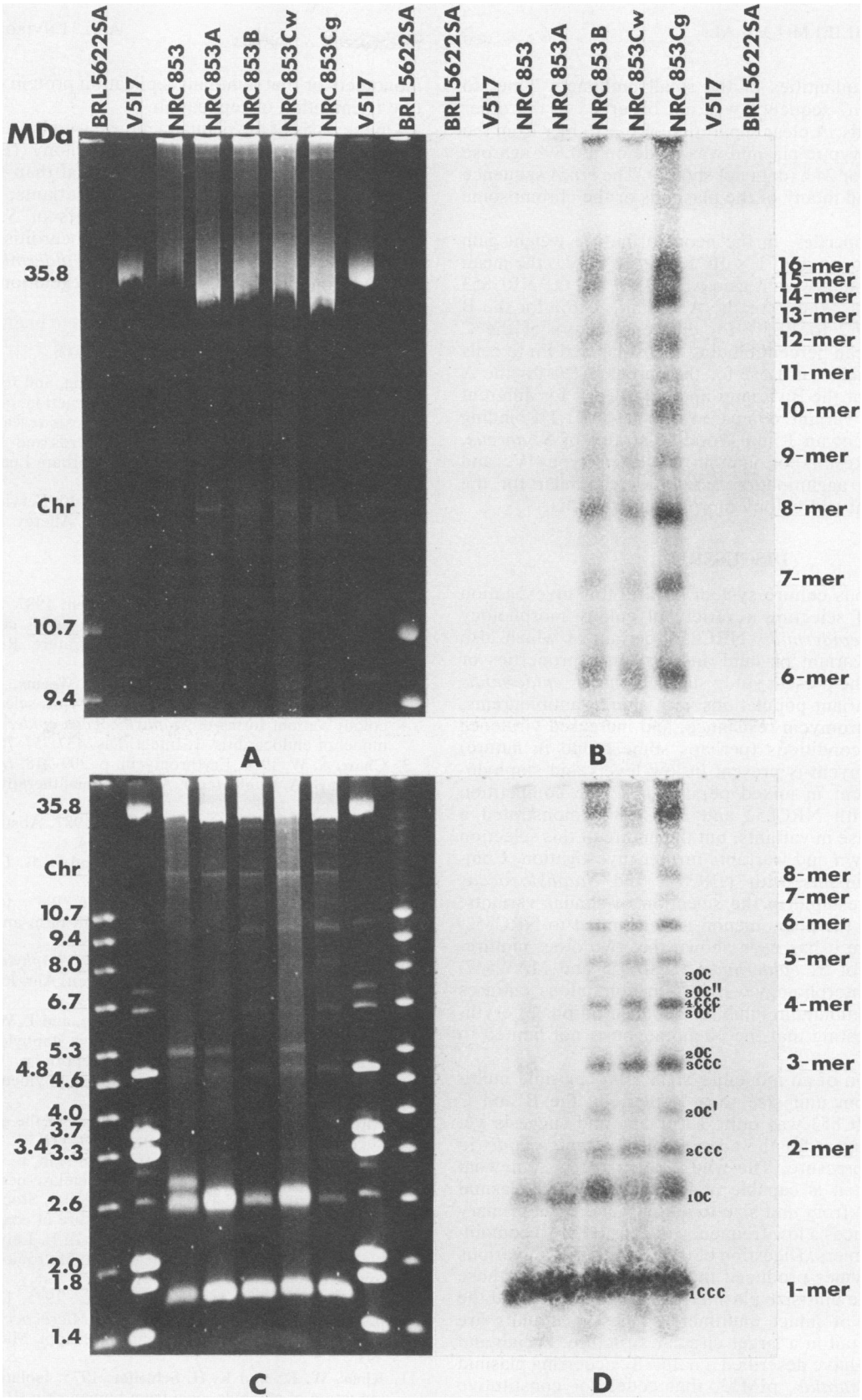

FIG. 3. Agarose gel electrophoresis and Southern blot analysis of *S. epidermidis* NRC853 and its colony morphology variant DNAs probed with a 644-bp *ermC* *Hin*fI-*Hin*cII fragment from pWE204. Lane designations: BRL5622SA, supercoiled DNA ladder; V517, *E. coli* V517 plasmids; NRC853, wild-type parent; NRC853A, A variant; NRC853B, B variant; NRC853Cw, C variant (white sector); NRC853Cg, C variant (gray sector). Panel designations: A, 0.8% agarose gel electrophoresis run at 30 V for 24 h; B, Southern blot of gel in panel A; C, 0.8% agarose gel electrophoresis run at 30 V for 11 h; D, Southern blot of gel in panel C. Covalently closed circular (CCC) and open circular (OC) forms of the 1- to 3-mer *ermC* plasmid are indicated in panel D. OC′ and OC″ forms probably represent incomplete open molecules.

but detectable quantities of the small multimers 2-mer to 4-mer. The *ermC* sequence was not observed in the other resident plasmids. A clear separation of the 16-mer from the large resident cryptic plasmid was made on a 0.8% agarose gel run at 50 V for 24 h (data not shown). The *ermA* sequence was not detected in any of the plasmids or the chromosome of NRC853.

Virulence properties. In the neonatal mouse weight gain test using an inoculum of 1×10^9 to 4×10^9 CFU, the mean percent decrease in weight gain was 14.0% for the NRC853 wild-type parent, 20.0% for the A variant, 35.6% for the B variant, and 17.2 to 19.5% for different sectors of the C variant. The mean percent binding of ^{125}I-labeled Fn to cells (5×10^9 CFU/ml) was 1.5% for the parent, 1.6% for the A variant, 4.4% for the B variant, and 1.0 to 1.6% for different sectors of the C variant, compared with 6.3 and 1.1% binding of Fn for the Cowan 1 and Wood 46 strains of *S. aureus*, respectively. Results of laminin, collagen type IV, and gelatin particle agglutination assays were similar for the wild-type parent and colony morphology variants.

DISCUSSION

The continuous culture system used in this investigation was capable of selecting a variety of colony morphology variants of *S. epidermidis* NRC853, certain of which also demonstrated variant plasmid and virulence properties or plasmid loss. The present study suggests that *S. epidermidis* can produce variant populations with altered antibiograms, increased erythromycin resistance, and increased virulence under certain conditions (perhaps some found in nature) where erythromycin is present in low levels and staphylococci are present in mixed populations. The competition experiments with NRC852 and NRC853 demonstrated a dramatic increase in variants, but the nature of this selection remains unknown and warrants further investigation. Competition experiments with NRC853 and *Staphylococcus warneri* have resulted in the selection of similar variants, suggesting that the phenomenon is not limited to NRC852, and furthermore it has been shown that two other multiresistant strains of *S. epidermidis* (DEM855 and MAW847) select colony morphology variants in continuous cultures exposed to subminimum inhibitory concentrations of erythromycin, suggesting that the phenomenon is not limited to NRC853 (3b).

The formation of an inducible MLS *ermC* plasmid multimer series, from unit size to a 16-mer, by the B and C variants of NRC853 was quite surprising and suggests yet another mechanism for the adaptation of *S. epidermidis* to erythromycin pressure. The wild-type parent is somewhat unusual in that it is capable of forming an *ermC* plasmid multimer series from unit size to a 4-mer and upon primary isolation, produces a low frequency of cells ($<10^{-4}$) containing larger multimers. Digestion of the multimers with various restriction enzymes produced the same fragments as those produced by the unit-size plasmid. This observation and the estimated size of intact multimers suggest that units are joined head to tail in a larger circular structure. Projan and coworkers (25) have described a naturally occurring plasmid from *Bacillus subtilis*, pIM13, that codes for constitutive expression of macrolide-lincosamide-streptogramin B resistance (*ermC'*) and also produces a multimer series. The plasmid pIM13 was transferred to *S. aureus*, and although it was unstable in this host it still produced multimers. These authors suggested that multimerization might occur by the loss of a site involved in the resolution of multimers into monomers or that a mutant replication protein causes defective termination of replication.

On the basis of the neonatal mouse weight gain test and Fn binding, it follows that the small-colony (B) variant of NRC853 has more pathogenic potential than the wild-type parent and other colony morphology variants. In support of this observation, small-colony variants of *S. epidermidis* were selected in the rat model of endocarditis (2). Furthermore, a small-colony variant of *S. epidermidis* has been isolated from the blood and resected vegetations of a patient with prosthetic valve endocarditis (1).

ACKNOWLEDGMENTS

We thank Hosni Hassan, Gene Namkoong, and Jerome Perry for their assistance and advice in the construction of a continuous culture system and review of the M.S. thesis research of the first author. We also are grateful to Alan Roberds and Angie Sorie for technical assistance and Jane Grant and Barbara Leasure for typing the manuscript.

This work was supported by Public Health Service grant 1 ROI AI21312 from the National Institute of Allergy and Infectious Diseases.

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