JOURNAL OF BACTERIOLOGY, Feb. 1982, p. 642–649
0021-9193/82/020642-08$02.00/0

Vol. 149, No. 2

Structural Analysis of Plasmid pSN2 in *Staphylococcus aureus*: No Involvement in Enterotoxin B Production

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Received 15 June 1981/Accepted 5 October 1981

Earlier studies have suggested the involvement of a small 1.3-kilobase plasmid, pSN2, in the production of enterotoxin B by certain *Staphylococcus aureus* strains. On the basis of extensive biochemical studies on pSN2, including the determination of its coding properties and its primary nucleotide sequence, we conclude that this plasmid is not in fact involved in enterotoxin B production in *S. aureus*: although the toxin genes are apparently chromosomal, it is probable that they are part of a special genetic system such as a hitchhiking transposon.

The first suggestion that there was anything unusual about the genetic control of enterotoxin B production in *Staphylococcus aureus* was contained in a paper by Dornbusch and Hallander (7), who reported that the determinant of this toxin in strain DU4916 was frequently transduced in association with methicillin resistance. The two determinants were themselves cotransduced either with a penicillinase plasmid or with a tetracycline resistance plasmid with varying frequencies (6, 27). Subsequent studies showed that these transductional associations did not lead to any detectable permanent change in the structure of either of these plasmids, and indeed, both the determinants of methicillin resistance and of enterotoxin B have recently been found to be chromosomal (25, 29). A further complication of these findings is contained in the results of Shalita et al. (27), who found that strain DU4916, originally studied by Dornbusch et al. (7), contained a small (1.3-kilobase [kb]) plasmid, pSN2, in addition to a tetracycline resistance plasmid (pSN1, 4.5 kb) and a penicillinase plasmid (pSN3, 30 kb). These investigators described genetic tests suggesting that the ability to produce enterotoxin B was associated with the small pSN2 plasmid: curing of the pSN2 by intercalating dyes or UV irradiation always resulted in loss of enterotoxin B production, whereas its reintroduction (by unselected cotransfer with pSN1) resulted in a restoration of the ability to produce the toxin. In addition, their results seemed to suggest that pSN2 is a satellite plasmid, i.e., that it can be maintained only in the presence of pSN1.

We undertook a series of experiments to evaluate the structure and coding properties of pSN2 with respect to the question of whether it contains the structural gene for enterotoxin B. We present here the complete nucleotide sequence of pSN2 DNA, its coding properties, and a series of experiments showing that pSN2 is not involved in enterotoxin B production. The earlier results probably were a reflection of an unusual type of mobility and lability of the enterotoxin B determinant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Table 1 lists the *S. aureus* strains and plasmids used. Bacterial strains were stored in CY broth at −75°C and were grown on GL agar or in CY broth (19) at 37°C (or at 32°C for plasmids that are temperature sensitive for replication [Tsr]). For enterotoxin B production, cells were grown in 6% NAK medium (5) for 24 h with shaking.

Restriction enzymes used were purchased from either New England Biolabs or Bethesda Research Laboratories. T4 DNA ligase was prepared by the method of Murray et al. (18). T4 polynucleotide kinase was a gift from Z. Humayun. Anti-enterotoxin B antiserum was kindly donated by M. Bergdoll and Z. Shalita. All other chemicals were from standard commercial sources. [γ-^{32}P]ATP was purchased from New England Nuclear Corp. (specific activity, 3,000 Ci/mmol). [α-^{32}P]dATP, -dTTP, and -dCTP were purchased from Amersham Corp. (specific activity, 2,000 Ci/mmol).

Isolation of plasmid DNA. Plasmid DNA was isolated by standard procedures involving dye-buoyant density centrifugation of cleared lysates (4, 21).

Transformation and enzymatic treatment of DNA. *S. aureus* strains were transformed by various plasmid DNAs by the protoplast transformation technique (3). The transformants were selected for the respective drug resistance markers and were screened for plasmid content by 0.7 or 1% agarose gel electrophoresis of sheared whole-cell lysostaphin lysates (8). Plasmid DNA was digested with restriction enzymes in the buffers specified by the manufacturers. Restriction digests were screened by 5% vertical slab polyacrylamide gel electrophoresis. Electrophoresis was performed with Tris-borate-EDTA buffer, pH 8.3 (9). Gels were stained with ethidium bromide and photographed with UV light. DNA fragments were recov-

TABLE 1. Bacterial strains and plasmids

Strain	Plasmid(s)	Phenotype[a]	Source or derivation
DU4916	pSN1, pSN2, pSN3	Pen^r Mec^r Tc^r	Naturally occurring (7)
RN450	None	None	8325-3 cured of ϕ13 (19)
RN3219	pSA5120	Cm^r	Recombinant, pSA5000 × pSA0301 (14)
RN3218	pSA5000	Cm^r	pT181 × pC221 (14, 15)
RN3123	pSA0301	Tc^r	Tsr mutant of pT181 (14)
SN3	None	None	27
SN6	None	None	27
SN17	pSN3	Pen^r	27
SK21	pSA5120, pSN3	Pen^r Cm^r	DU4916 transformed with pSA5120; cured of pSN1 and pSN2
SK22	pSA5120, pSN2, pSN3	Pen^r Cm^r	DU4916 transformed with pSA5120; cured of pSN1
SK25	pSN1, pSN3	Pen^r Tc^r	DU4916 transformed with pSA5120::pSN2 cointegrate and grown at 43°C
SK26	pSN1, pSN3	Pen^r Tc^r	DU4916 transformed with pSA5120::pSN2 cointegrate and grown at 43°C
SK31	pSN2	None	RN450 transformed with pSA0301 + pSN2 and cured of pSA0301
COL	pSN1	Tc^r, Mec^r	26

[a] Tc^r = Tetracycline resistant; Cm^r = chloramphenicol resistant; Pen^r = Penicillin resistant; Mec^r = Methicillin resistant; Tsr = temperature sensitive for replication.

ered from the gels after electrophoresis as described (12).

Restriction fragments were treated with DNA polymerase I in the presence of deoxynucleotide triphosphates to fill in cohesive ends as described (1). These were used for cloning by blunt-end ligation with T4 ligase (1), and clones were then recovered by protoplast transformation.

Ends of restriction fragments were labeled, after dephosphorylation with bacterial alkaline phosphatase, by rephosphorylation in the presence of [γ-^{32}P]ATP and polynucleotide kinase (12). After digestion with appropriate restriction enzymes, the 5'-end-labeled fragments were purified by polyacrylamide gel electrophoresis and autoradiographed by exposure to Kodak-RP Royal X-Omat X-ray film.

DNA sequence determination. DNA sequencing reactions were done essentially as described by Maxam and Gilbert (17). Dimethylsulfate was used for guanine (G) reactions; pyridinium formate was used for adenine-plus-guanine (A + G) reactions; hydrazine was used for cytosine-plus-thymidine (C + T) reactions; and hydrazine in the presence of 5 M NaCl was used for C reactions. Final cleavage products were run on 40 by 0.04 cm, 20% acrylamide–8.3 M urea gels (for the first 20 to 30 nucleotides) and 8% acrylamide–8.3 M urea gels for sequences between 30 and 200 nucleotides. Both of the DNA strands were sequenced.

DNA-DNA hybridization. pSN2 DNA was labeled with ^{32}P by nick translation with DNA polymerase I (23) and ^{32}P-labeled dTTP, dATP, and dCTP. The specific activity was 4×10^7 cpm/μg. Cells were lysed with lysostaphin (21) and, after sodium dodecyl sulfate (SDS) treatment, 4 μl of lysates from various strains was applied to a nitrocellulose filter. Hybridization was carried out with 10^6 cpm of ^{32}P-labeled pSN2 DNA in 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 16 h at 55°C, and the filter was autoradiographed (10).

Double-diffusion analysis. Cultures were obtained for enterotoxin B production by growing strains at 37 or 32°C for 16 to 24 h with shaking in 6% NAK medium as described (5). Supernatants were subjected to double diffusion in agar against anti-enterotoxin B antiserum (5).

Preparation of cell-free extracts and protein synthesis. Cell-free extracts from *Escherichia coli* and *S. aureus* were prepared, and protein synthesis was carried out as described (16, 31). Reactions were done in a final volume of 100 μl, and 30 μl of S-30 was added to each tube. pSN2 DNA concentration was 10 μg/ml. After incubation at 37°C for 40 min, 0.5 volume of "cracking buffer" was added; then 3-μl samples were heated at 100°C for 2 min, and ^{3}H-labeled products were run on 15% SDS-polyacrylamide slab gels (30). Protein synthesis in minicells was carried out with purified minicells from *Bacillus subtilis* strain CU403 containing the plasmid(s) to be tested. Synthesis and analysis were done as described (28). ^{14}C-labeled proteins used as molecular weight standards were obtained from New England Nuclear. After 2,5-diphenyloxazole (PPO)-dimethyl sulfoxide treatment, the gel was dried and exposed to Kodak X-Omat R film for 2 to 3 days at −70°C (2).

RESULTS

Transformation with pSN2. Since pSN2 does not contain a selectable marker, transformants were obtained by congression. A mixture of pSN1 and pSN2 DNAs at a weight ratio of 1:100 was added to a suspension of protoplasts of the required recipient in the presence of polyethylene glycol 6000; following the usual procedure, the protoplasts were incubated in SMMP (24) for 4 h to permit the phenotypic expression of the pSN1 tetracycline resistance (Tc^r) marker. Virtually all of the transformants selected for tetracycline resistance under these conditions contained pSN2 as well as pSN1 (Fig. 1). By this means, pSN2 was introduced into RN450, a

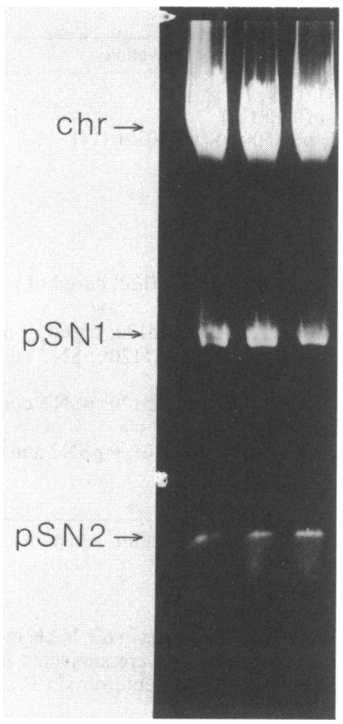

FIG. 1. One percent agarose gel analysis of RN450 derivatives obtained by transformation with pSN1 and pSN2 DNAs.

nontoxigenic *S. aureus* strain, and CU403, the minicell-producing *B. subtilis* strain. When supernatants of such pSN2-containing strains were analyzed by double-diffusion agar in comparison with the original donor, DU4916, it was evident that they failed to produce detectable enterotoxin B (Fig. 2).

Protein synthesis in vitro and by minicells. Figure 3 shows the results of in vitro protein synthesis and minicell protein synthesis directed by the pSN2 plasmid. pSN2 coded for a single polypeptide of 20 kilodaltons (kdal) in minicells (compare lanes e and f). This did not correspond to enterotoxin B, which has a molecular size of 29 kdal (11). Coupled transcription-translation of pSN2 in both *E. coli* and *S. aureus* extracts also resulted in synthesis of a 20-kdal polypeptide (lanes a through c); in addition, a 12-kdal polypeptide was produced. Again, there was no 29-kdal species that might correspond to enterotoxin B. The general agreement of these results leads us to conclude that the gel electrophoresis patterns are probably a correct representation of the coding properties of pSN2, and therefore the plasmid does not carry the structural gene for enterotoxin B.

At this stage there seemed to be three possibilities: (i) pSN2 encodes part of the enterotoxin B polypeptide and the chromosome encodes the rest; (ii) pSN2 is required for enterotoxin B synthesis by an unlinked structural gene; or (iii) the previous inferences of pSN2 involvement are erroneous. To distinguish among these possibilities, we determined the complete nucleotide sequence of the plasmid and undertook to cure the pSN2 plasmid by means that avoided subjecting the organisms to mutagenic "curing" procedures such as treatment with intercalating dyes or UV radiation.

Structural organization of pSN2. pSN2 DNA was first mapped by digestion with various restriction enzymes, singly and in pairs, and the results of this series of analyses are presented as a restriction map in Fig. 4. The primary nucleotide sequence of the entire plasmid was determined by the Maxam-Gilbert method (17), according to the sequencing strategy shown in Fig. 5. The entire sequence is shown in Fig. 6. Computer analysis of this sequence revealed a coding sequence extending from positions 701 to 277. This sequence encoded a polypeptide of 158 amino acids with a molecular size of about 20 kdal which we assume corresponds to the single protein species in minicells. The coding sequence for this polypeptide was flanked by sequences that are typically associated with translatable regions in other systems. Thus, there was a good Pribnow sequence at position 745 and an RNA polymerase recognition site at position 789 (22). The putative mRNA that would initiate at this site could terminate at a G + C-rich stem which is followed by a possible mRNA temination site T_6A at position 171.

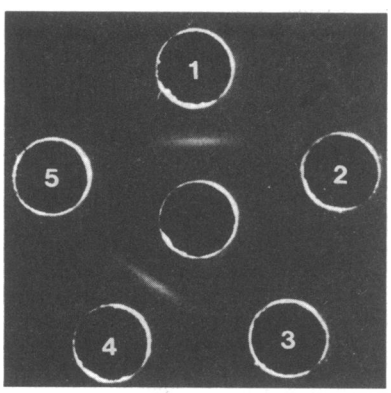

FIG. 2. Immunodiffusion analysis of enterotoxin B production by various pSN2-containing strains. Various transformants were grown in NAK medium, and culture supernatants were analyzed for the presence of enterotoxin B as described (7). Center well contained anti-enterotoxin B serum. Outer wells: (1) enterotoxin B; (2) RN450 supernatant; (3) RN450 containing pSN1 and pSN2 supernatant; (4) DU4916 supernatant; (5) CU403 containing pSN1 and pSN2 supernatant.

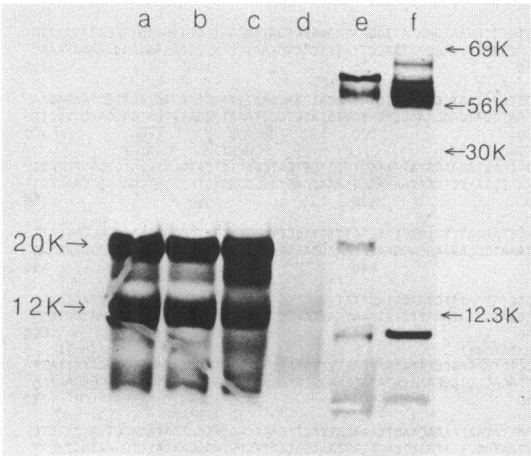

FIG. 3. Fluorography of a 15% SDS-polyacrylamide slab gel with ^{3}H-labeled products of in vitro protein synthesis, using pSN2 DNA and *B. subtilis* minicell protein synthesis. (a–d) In vitro protein synthesis. (a) *S. aureus* extract; (b) *E. coli* extract; (c) *E. coli* extract and *Hpa*II-linearized pSN2 DNA; (d) *E. coli* extract and *Taq*I-linearized pSN2 DNA. (e) Minicell protein synthesis with pSN1 and pSN2 DNAs; (f) minicell synthesis with pSN1 DNA. Positions of standard molecular weight markers are shown by arrows.

There was a strong ribosomal binding site at position 715 followed by an appropriate initiation codon, ATG, at position 701, and the first chain-terminating codon after this was the TAG at position 227. There was no separate coding sequence that could possibly have given rise to the 12-kdal polypeptide observed after pSN2-directed transcription-translation in extracts of *E. coli* and *S. aureus*. We assume, therefore, that this polypeptide must represent internal initiation within the larger coding sequence just described. In support of this conclusion, we observed that pSN2 DNA linearized with *Taq*I, which recognized a single site at position 301, did not give any detectable products in the coupled in vitro system, whereas both the 20- and 12-kdal polypeptides were synthesized when the system was programed by *Hpa*II-linearized DNA (Fig. 3). The amino acid sequence of the 20-kdal protein deduced from the DNA sequence bore no resemblance whatsoever to the amino acid sequence of enterotoxin B (11). This result effectively rules out alternative (i) above.

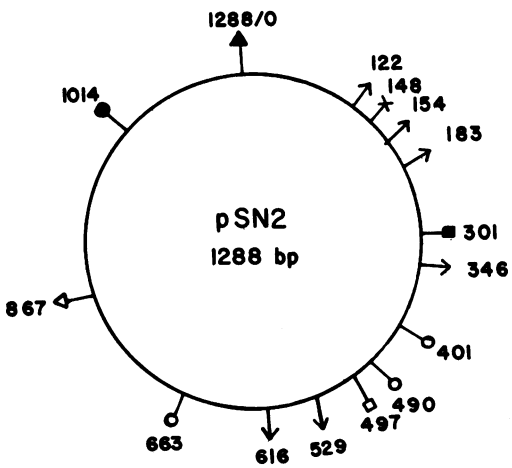

FIG. 4. Restriction map of the pSN2 plasmid. The index point of the map is the single *Hpa*II site. Coordinate numbers refer to base pairs. Symbols: →, *Alu*I; ——○, *Dde*I; ——●, *Fnu*4HI; ——▷, *Hin*fI; ——▶, *Hpa*II; ——□, *Rsa*I; ——■, *Taq*I; ——X, *Mbo*I.

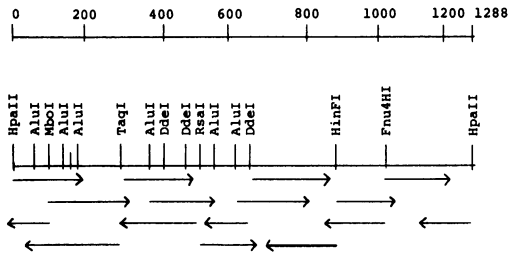

FIG. 5. Sequencing strategy for pSN2 DNA. Both the strands were sequenced. Numbers refer to base pairs. Arrows indicate the direction and extent of sequence determination.

Possible role of pSN2 in the control of enterotoxin B synthesis. It was reported that derivatives of DU4916 cured of pSN2 also lost the ability to produce enterotoxin B and that their ability to produce the toxin was restored upon reintroduction of pSN2 by transduction (27). Since pSN2 does not contain information for enterotoxin B structure, its only possible role would have to be a requirement for the expression of an unlinked (i.e., chromosomal) structural enterotoxin B determinant. Several derivatives of DU4916 cured of pSN2 and unable to synthesize enterotoxin B were kindly provided by Z. Shalita. These were verified as enterotoxin B minus by immunodiffusion and were then transformed with pSN2 by congression with pSN1 and selection for tetracycline resistance. Transformants with either pSN1 alone or with both plasmids were obtained, and immunodiffusion tests revealed no detectable enterotoxin B antigen (data not shown).

This confirmed our conclusion that pSN2 does not contain the structural gene for enterotoxin B and suggested that the DU4916 derivatives did not contain the gene either. We concluded tentatively that the structural gene for enterotoxin B, although evidently chromosomally located, was either genetically or functionally "labile" and

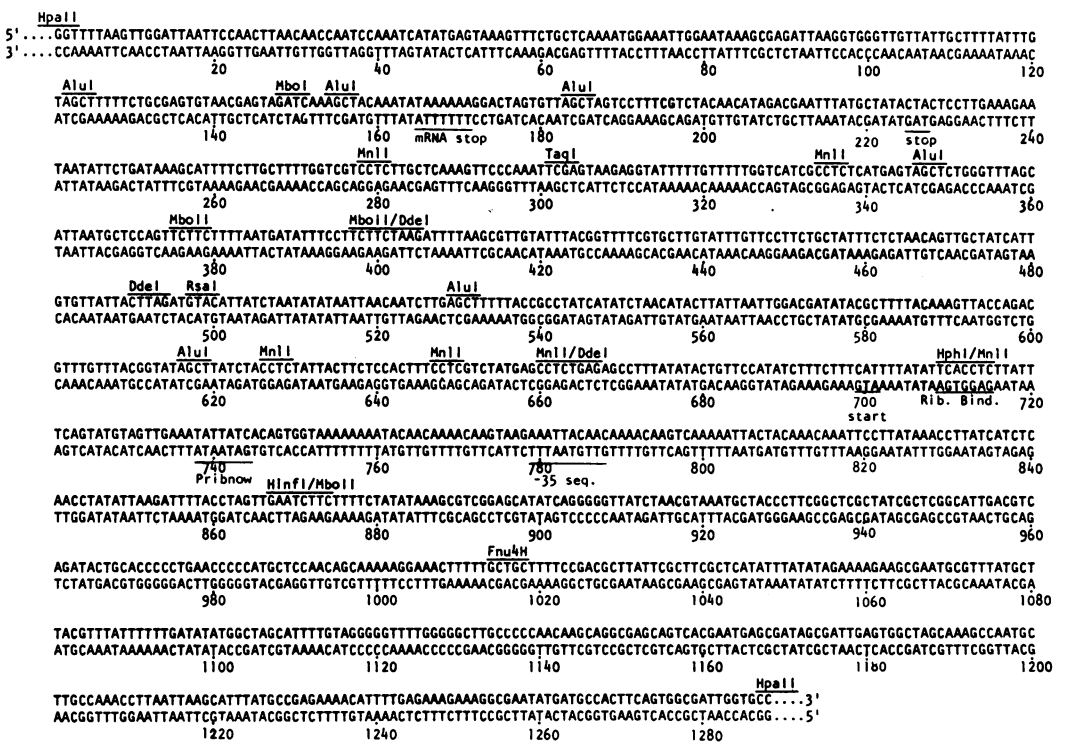

FIG. 6. Complete nucleotide sequence of pSN2 DNA. The Pribnow box, −35 sequence, ribosomal binding site and the presumed sites for mRNA termination, and start and stop sites for the 20-kdal polypeptide are shown, as are the known restriction sites.

had been eliminated or inactivated concomitantly with the elimination of pSN2 by Shalita et al. (27). A test of this possibility required the elimination of pSN2 from an enterotoxin B-producing strain by a curing process that avoided treatment with toxic chemicals and other damaging agents such as UV radiation.

pSN2 was cloned into carrier plasmid pSA5120, formed by recombination between pSA5000, a chloramphenicol resistance (Cm^r) in vivo recombinant between pC221 and pT181 (14, 15) and pSA0301, a mutant of pT181 that carries the thermosensitive *repC1* allele (13). pSA5120 carries this Cm^r marker and has the Tsr *repC1* allele of pSA0301 and the Inc3 phenotype of pT181. The pSA5120-pSN2 clone could then be used to displace the resident pSN2 plasmid by incompatibility. The clone would then be eliminated either by elimination at the restrictive temperature (if the pSN2 replication was inactivated by the insertion of pSA5120) or by incompatibility with the resident pSN1 (Tc^r Inc3) plasmid. The cloning was done by digesting pSA5120 with *Hin*dIII and pSN2 with either *Taq*I or *Mbo*I. After the cohesive ends were filled with polymerase I and deoxyribonucleotide triphosphates, pSA5120 and pSN2 were blunt-end ligated with T4 DNA ligase at 14°C overnight. The composite plasmids were introduced by protoplast transformation into DU4916, with selection for chloramphenicol resistance. Several transformants were screened by agarose gel electrophoresis of sheared whole-cell lysates. Many had lost the pSN2 plasmid (Fig. 7).

Several of the transformants containing the pSA5120::pSN2 composites were then plated on nonselective medium, grown overnight at 43°C, and scored for loss of the composite by replica plating to chloramphenicol-containing agar. Chloramphenicol-susceptible colonies were recovered at a frequency of approximately 1%, indicating that the composites were only minimally, if at all, thermosensitive for replication. The same result was obtained for *Taq*I- and *Mbo*I-generated clones. We noted that all of the chloramphenicol-susceptible colonies were tetracycline resistant and were found by agarose gel electrophoresis to have retained a plasmid species with the same mobility as pSN1, the Tc^r plasmid originally present in DU4916. On the basis of these results, it was clear that there was at most a very mild incompatibility which has been subsequently confirmed directly (A. Gruss,

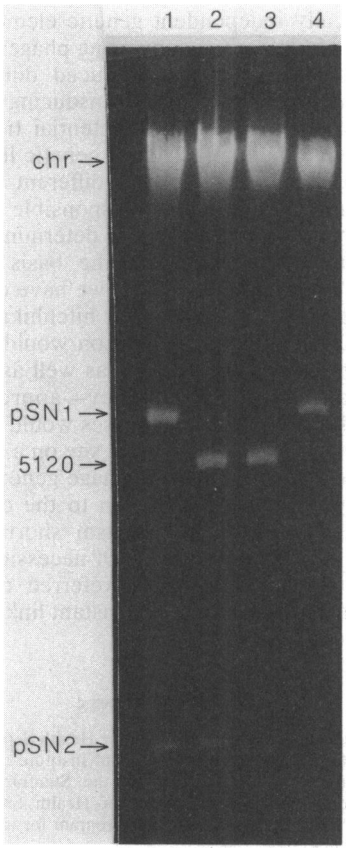

FIG. 7. One percent agarose gel analysis of strain DU4916 and derivatives. (1) DU4916; (2) DU4916 containing pSA5120 and pSN2 and cured of pSN1 (SK22); (3) SK21; (4) SK25.

personal communication). We concluded that this incompatibility was probably responsible for the loss of the composite plasmid. It is also possible that this plasmid is inherently slightly unstable; this has not been tested.

Finally, various sets of these derivatives were analyzed for enterotoxin B production by immunodiffusion (Fig. 8), and all were positive, irrespective of their plasmid content.

To rule out the possibility of the integration of the pSN2 plasmid into the host chromosome, we carried out DNA-DNA hybridization experiments. As shown in Fig. 9, none of the pSN2 negative strains had pSN2 sequence integrated into the host chromosome.

Maintenance of pSN2. pSN2 does not require any helper plasmid for maintenance and replication in *S. aureus*. We cotransformed protoplasts of *S. aureus* strain RN450 with a Tsr tetracycline resistance plasmid, pSA0301 (13), and pSN2 DNAs. Tetracycline-resistant transformants containing both pSA0301 and pSN2 were identified by gel electrophoresis and cured of pSA0301

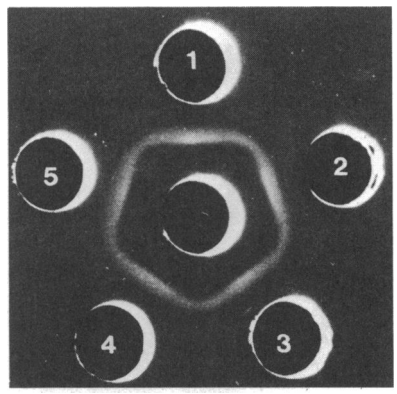

FIG. 8. Immunodiffusion analysis of enterotoxin B production by various DU4916 derivatives. Center well contained the anti-enterotoxin B serum. Outer wells: (1) DU4916; (2) DU4916 containing pSA5120 (SK21); (3) DU4916 containing pSA5120 and pSN2 (SK22); (4) DU4916 cured of pSN2 (SK25); (5) DU4916 cured of pSN1 and pSN2 and containing pSA5120 (SK26).

by growing overnight at 43°C on nonselective medium. All of the tetracycline-susceptible colonies that were screened had lost pSA0301 but retained pSN2, whereas tetracycline-resistant colonies had retained both plasmids (Fig. 10).

DISCUSSION

We have shown that derivatives of DU4916 that are cured of pSN2 still produce enterotoxin

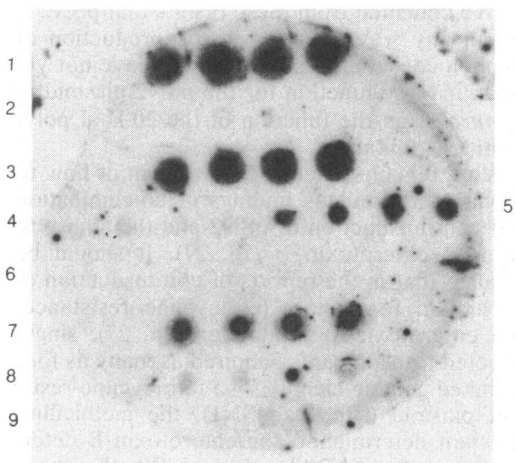

FIG. 9. DNA-DNA colony hybridization (four colonies each) of various strains with pSN2 DNA. Whole-cell lysates from various strains were applied to a nitrocellulose filter, and 10^6 cpm of ^{32}P-labeled pSN2 DNA was then hybridized to the filter. (1) SK31; (2) SK26; (3) SK22; (4) SK21; (5) RN450 overlayed with 2 ng of pSN2 DNA; (6) SK25; (7) 834; (8) RN450; (9) COL. Longer exposures of the filter did not show any further detectable hybridization.

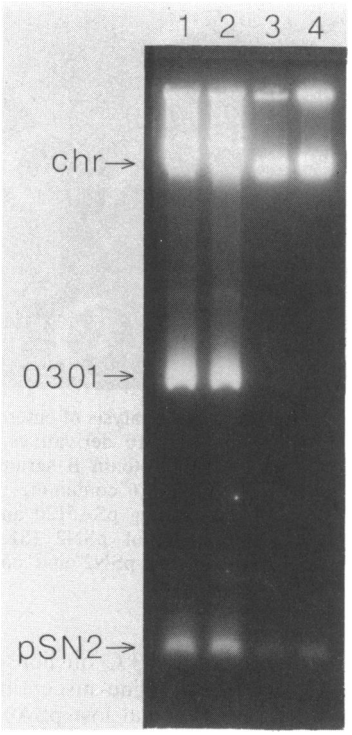

FIG. 10. One percent agarose gel analysis of double transformants cured of pSA0301 plasmid. (1) RN450 containing pSA0301 and pSN2 plasmids; (2) tetracycline-resistant colony; (3 and 4) tetracycline-sensitive colonies.

B. We conclude from these results that pSN2 is not in any way involved in the production of staphylococcal enterotoxin B. We have not yet identified any function for the pSN2 plasmid in *S. aureus*, as the function of the 20-kdal polypeptide is unknown.

What remains, then is the problem of how to explain the previous findings of co-elimination and contransduction of pSN2 and the ability to produce enterotoxin B (26, 27). It should be recalled that in the reports of cotransduction of methicillin resistance, tetracycline resistance, and enterotoxin B production (26, 27), singly selected transductants acquired as many as four unlinked genetic elements—a tetracycline-resistant plasmid (such as pSN1), the methicillin-resistant determinant, the enterotoxin B determinant, and a pSN2-like plasmid. Whether these four elements can all be packaged by a single phage particle, it is clear that unusual transfer systems are involved, and it would seem appropriate to consider for further study converting phages (possibly defective) and hitchhiking transposons (21).

The previous results might have been a consequence of frequent co-elimination or cotransfer of physically independent genetic elements. In the case of (defective) converting phages, infective particles would be produced during the vegetative growth of the transducing phage. These would then coinfect potential transductants, giving the impression of genetic linkage.

Alternatively, a somewhat different type of genetic mobility might be responsible for the behavior of the enterotoxin B determinant and other similar elements. On the basis of our analysis of transposon Tn*554*, we have come to refer to this type of mobility as hitchhiking (20). Here, the hitchhiking transposon would have a high degree of site specificity as well as a very high transposition frequency—approaching 100%. Mobilization in such cases would involve transposition to an appropriate site on a suitable carrier replicon (plasmid or phage genome) followed by second transposition to the chromosome of the recipient organism shortly after entry of the carrier replicon. Of necessity, such transposons have a highly preferred chromosomal site, which results in constant linkage and may obscure their true nature.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI 17216 (to S.A.K.) from the National Institute of Allergy and Infectious Diseases. We thank the Stanford Molgen project and the National Institutes of Health Sumex-Aim Facility for the use of the computer program for analysis of DNA sequences.

We thank T. Aldrich and H. Ali for excellent technical assistance.

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