10 percent oxygen (*13*), indicating that mudminnows may have been able to obtain sufficient oxygen more quickly at bubbles with 20 percent oxygen.

Selection for the ability to use gaseous oxygen would be intense during the hypoxic and hypercarbic conditions of winter. Fish using gaseous respiration not only obtain more oxygen but can also avoid high amounts of dissolved hydrogen sulfide, which can reach lethal concentrations in winterkill lakes (*4, 5, 14*). Although selection pressures for gaseous respiration are obviously great in winter, air-breathing by mudminnows might have evolved as an adaptation to low *p*H conditions in summer. Mudminnows in northern Wisconsin often inhabit waters with moderately low *p*H (*15*). In experiments examining effects of *p*H and elevated temperatures on air-breathing in mudminnows, there were no statistically significant differences ($P \geq .05$) observed in rates of air-breathing among fish held in water from a dystrophic (*p*H 4.5), oligotrophic (*p*H 5.6), or mesotrophic (*p*H 6.8) lake (0.7, 1.4, and 0.9 breaths per fish per hour, respectively) (*6*). Also, frequency of air-breathing did not increase significantly ($P \geq .05$) as water temperatures increased from 16°C to a lethal level (34°C) regardless of the type of lake water (*16*).

By using the gaseous oxygen in bubbles, mudminnows are able to survive concentrations of DO an order of magnitude lower than those reported as stressful to most fishes that rely on aquatic respiration (*17*). Moreover, the ability of mudminnows to respond differently to bubbles varying in oxygen content would enhance their ability to survive in winterkill lakes, where bubbles differ greatly in composition. Whether the bubbles come from sediments, from gases extruded in water when it freezes, or from gases exhaled or escaping from the fur of aquatic mammals, they are a critical winter resource for mudminnows, allowing them to survive in environments that are lethal to other fishes.

JOHN J. MAGNUSON
JOHN W. KELLER
ANNAMARIE L. BECKEL
GEORGE W. GALLEPP
Center for Limnology and
Department of Zoology, University of Wisconsin, Madison 53706

References and Notes

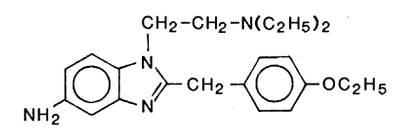
1. J. H. Gee, *Can. J. Zool.* **58**, 819 (1980).
2. K. Johansen, in *Fish Physiology*, W. S. Hoar and D. J. Randall, Eds. (Academic Press, New York, 1970), vol. 4, p. 361; E. N. Willmer, *J. Exp. Biol.* **10**, 283 (1933).
3. S. A. Klinger, J. J. Magnuson, G. W. Gallepp, *Environ. Biol. Fish.* **7**, 113 (1982).
4. J. J. Magnuson and D. J. Karlen, *J. Fish. Res. Board Can.* **27**, 1059 (1970).
5. W. M. Tonn and J. J. Magnuson, *Ecology* **63**, 1149 (1982).
6. Lake water (Trout Lake, Vilas County, Wisconsin), passed through two columns for stripping oxygen with nitrogen gas, was delivered to each aquarium at a flow rate of 250 to 300 ml/min; CO_2 was added in the second column. Stripping is described by Petrosky and Magnuson (*11*).
7. The observer (in an observation area with low light intensity) viewed the fish through a one-way mirror set at an angle. Observations were usually made between 0915 and 1445. Each aquarium was lit by incandescent bulbs controlled by timers with day length simulated for winter conditions.
8. Temperature and chemical conditions were measured at least once a day in each aquarium from 2 hours before to 1/2 hour after observations. Water samples were siphoned from the aquariums; DO was measured by Winkler titration (azide modification) and CO_2 by titration with sodium carbonate. Replicate chemical measurements made on 3 days agreed closely.
9. J. H. Gee, *J. Exp. Biol.* **92**, 37 (1981).
10. K. H. Mills, thesis, University of Wisconsin, Madison (1972).
11. B. R. Petrosky and J. J. Magnuson, *Copeia* **1973**, 124 (1973).
12. Kruskal-Wallis rank sum test for multiple comparisons.
13. Range test for Kruskal-Wallis rank sum test for multiple comparisons.
14. L. L. Smith, Jr., *et al.*, *Trans. Am. Fish. Soc.* **105**, 442 (1976); L. L. Smith, Jr., D. M. Oseid, L. E. Olson, *Environ. Sci. Technol.* **10**, 565 (1976).
15. *p*H 4.0 to 7.0 [F. J. Rahel and J. J. Magnuson, *Can. J. Fish. Aquat. Sci.* **40**, 3 (1983)].
16. J. J. Magnuson and A. L. Beckel, unpublished observations. Six mudminnows were held in each of four aquariums (two of *p*H 4.5, one of *p*H 5.6, and one of *p*H 6.8), as water temperature increased for 13 days from 16° to 34°C. Each aquarium was observed for 30 minutes each day. The Kruskal-Wallis rank sum test for multiple comparisons was used for all analyses.
17. J. C. Davis, *J. Fish. Res. Board Can.* **32**, 2295 (1975).
18. Mudminnows were collected from Mann Creek flowage in Vilas County, Wisconsin, on 23 January 1972. Three fish died during the experiments.
19. In each experiment the positions of bubbles, open holes, and plugs were changed randomly between each 30-minute observation period.
20. We thank S. Steffel, R. Ozane, R. Stein, B. Richardson, S. Brandt, S. Petty, and S. Klinger for assistance in observations or analyses. Supported by NSF grant GB 18272.

2 August 1982; revised 6 December 1982

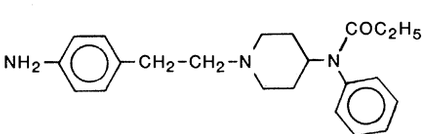
Irreversible Ligands with High Selectivity Toward δ or μ Opiate Receptors

Abstract. *Alkylating agents that display strong selectivity for opiate receptor types δ or μ were prepared by appropriate modification of the structures of the strong analgesics fentanyl, etonitazene, and endoethenotetrahydrooripavine. The availability of these substances should facilitate studies of the structural basis of receptor specificity and of the physiologic roles of these receptors.*

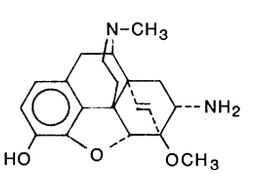
The existence of several endogenous opioid peptides (*1*) and data suggestive of separate types of opiate receptors (*2*) have raised questions about the relations of the peptides to the receptors and about the structure and function of the various types of receptors. Of the opiate receptor types so far proposed, δ and μ are the best documented (*2*) and together account for 70 percent of the opiate receptors of rat brain (*3*).



1



2



3

One approach to the study of these questions requires selective, site-directed alkylating agents for each receptor type. Probes with exclusive specificity for δ receptors have been heretofore unavailable, although several affinity reagents based on enkephalin have been prepared (*4*). In this report, we describe alkylating derivatives of several opiate analgesics and show that some of these are highly selective, and irreversible, ligands for δ receptors, whereas others are equally selective for μ receptors.

Irreversible ligands with apparent specificity toward μ (and perhaps κ) receptors have been prepared by modification of epoxymorphinan structures characteristic of morphine, the prototypical μ ligand (*5*). We used amino derivatives of strong analgesics based on etonitazene (structure **1**), fentanyl (structure **2**), and endoethenotetrahydrooripavine (structure **3**) to prepare alkylating ligands of greater selectivity. These amines were converted to the corresponding isothiocyanato-, bromoacetamido-, and methylfuramido- derivatives and assayed for covalent interaction with receptors. Opiate receptor assays were carried out with rat brain membranes, which have μ and δ receptors and with membranes from the neuroblastoma-glioma hybrid NG108-15 cells, which have only δ receptors (*6*).

Inactivation of δ receptors was measured after a 30-minute incubation of NG108-15 cell membranes with concentrations of alkylating ligand near their median inhibitory values. The number of functional opiate receptors remaining in the washed membranes was evaluated by Scatchard analysis of the binding of ^{3}H-labeled [D-Ala^2,Met^5]enkephalinamide (DALAMID). Low concentrations of several of the opiates reduced the number of receptors to fewer than 50 percent of control values (Fig. 1A); other opiates were much less effective. *N*-Phenyl-*N*-[1-(2-(*p*-isothiocyano)phenylethyl)-4-piperidinyl]propanamide (fentanyl isothiocyanate, FIT) and 7α-methylfumaramido-6,14-endoethenotetrahydrooripavine (fumaramido oripavine, FAO) are the two most potent agents and were chosen for further study along with the etonitazene derivative, 2-(*p*-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanobenzimidazole isothiocyanate (BIT), which shares with its parent compound a high affinity for μ receptors (see below).

We used etonitazene displacement of [^{3}H]DALAMID from brain membranes to measure δ and μ receptor binding. Etonitazene displaced approximately 60 percent of specifically bound [^{3}H]DALAMID at nanomolar concentrations [dissociation constant (K_d), 4 n*M*], whereas the remaining 40 percent of specifically bound ligand was not displaced until etonitazene was added at micromolar concentrations (K_d, 0.3 μ*M*) (Fig. 1B). DALAMID binds equally well to μ and δ receptors (*7*) and not at all to κ. When [^{3}H]morphine, a μ-selective ligand, is used in such experiments, etonitazene displacement occurs with nanomolar affinity, and with ^{3}H-labeled [D-Ala^2,D-Leu^5] enkephalin (a δ-selective ligand), etonitazene displacement occurs mainly with micromolar affinity (Fig. 1B). Thus, the receptors with high affinity for etonitazene are μ and those with low affinity are δ. The assignment is consistent with earlier observations that rat brain contains μ and δ opiate receptors in an approximately 3:2 ratio (*3, 6*). The assignment is also consistent with the fact that etonitazene displaces [^{3}H]DALAMID from the δ receptors of NG108-15 cell membranes in a monotonic fashion with a K_d of 0.1 μ*M*.

Table 1. Receptors remaining after treatment with alkylating opiates. Values are means ± standard error from triplicate experiments (one of which is shown in Fig. 1C). Numbers of receptors were calculated from the differences between the plateau values of the etonitazene competition curves.

Opiate	Receptors remaining (percent of control)	
	μ	δ
None	100 ± 3	100 ± 6
BIT	55 ± 3	96 ± 8
FIT	109 ± 8	26 ± 6
FAO	109 ± 9	44 ± 4

Rat brain membranes were incubated for 30 minutes at 37°C with 20 n*M* FIT or BIT or with 50 n*M* FAO. After the membranes were washed, the numbers of μ and δ receptors remaining were estimated by monitoring the displacement of [^{3}H]DALAMID binding by etonitazene (Fig. 1C). Only μ receptors were inactivated by BIT, and only δ receptors were inactivated by FIT and FAO. A quantitative assessment of the number of μ and δ receptors remaining from three independent measurements is presented in Table 1. Within the limits of experimental uncertainty, the data show no evidence for cross-reactivity. Thus, FIT and FAO are highly selective alkylators of δ receptors and BIT is equally highly selective toward μ.

Type-specific ligands in the form of ^{3}H-labeled compounds of high specific activity should facilitate studies of the structural basis of receptor subtype specificity and may clarify the physiologic roles of these receptors. Preliminary

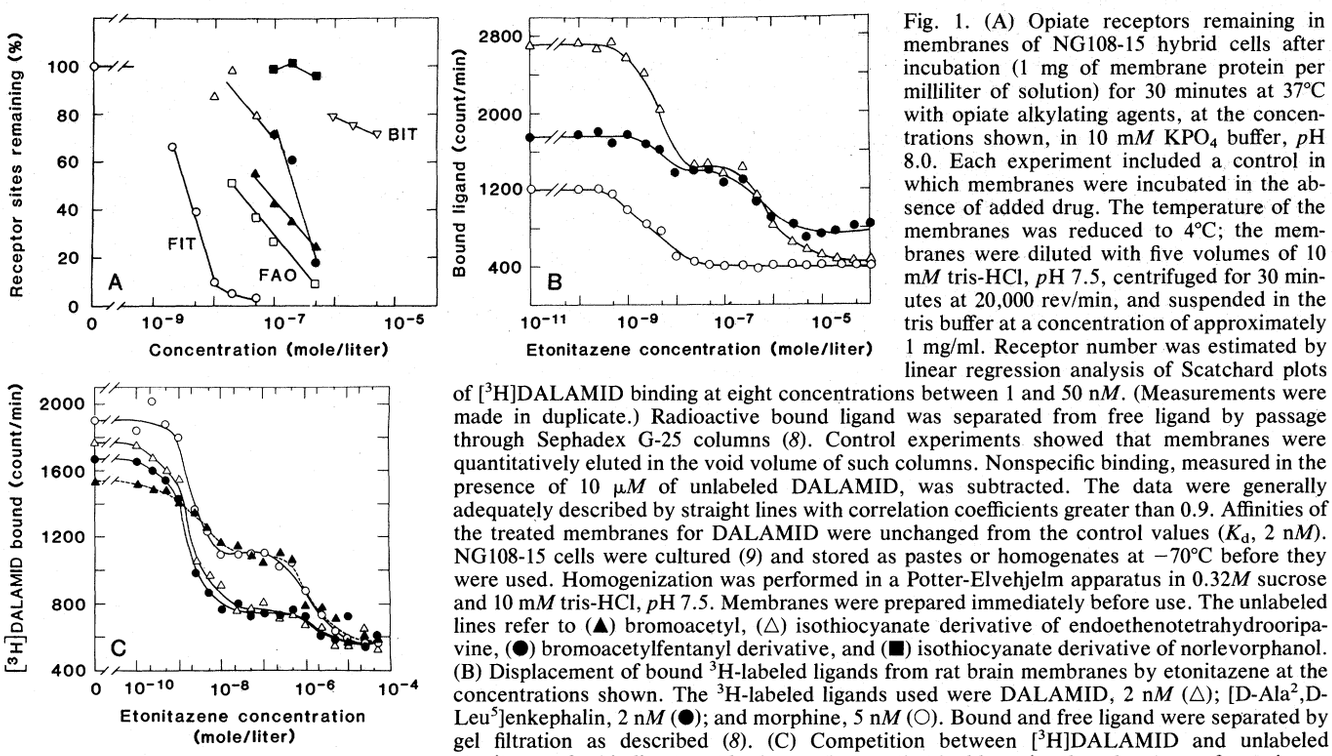


Fig. 1. (A) Opiate receptors remaining in membranes of NG108-15 hybrid cells after incubation (1 mg of membrane protein per milliliter of solution) for 30 minutes at 37°C with opiate alkylating agents, at the concentrations shown, in 10 m*M* KPO_4 buffer, *p*H 8.0. Each experiment included a control in which membranes were incubated in the absence of added drug. The temperature of the membranes was reduced to 4°C; the membranes were diluted with five volumes of 10 m*M* tris-HCl, *p*H 7.5, centrifuged for 30 minutes at 20,000 rev/min, and suspended in the tris buffer at a concentration of approximately 1 mg/ml. Receptor number was estimated by linear regression analysis of Scatchard plots of [^{3}H]DALAMID binding at eight concentrations between 1 and 50 n*M*. (Measurements were made in duplicate.) Radioactive bound ligand was separated from free ligand by passage through Sephadex G-25 columns (*8*). Control experiments showed that membranes were quantitatively eluted in the void volume of such columns. Nonspecific binding, measured in the presence of 10 μ*M* of unlabeled DALAMID, was subtracted. The data were generally adequately described by straight lines with correlation coefficients greater than 0.9. Affinities of the treated membranes for DALAMID were unchanged from the control values (K_d, 2 n*M*). NG108-15 cells were cultured (*9*) and stored as pastes or homogenates at −70°C before they were used. Homogenization was performed in a Potter-Elvehjelm apparatus in 0.32*M* sucrose and 10 m*M* tris-HCl, *p*H 7.5. Membranes were prepared immediately before use. The unlabeled lines refer to (▲) bromoacetyl, (△) isothiocyanate derivative of endoethenotetrahydrooripavine, (●) bromoacetylfentanyl derivative, and (■) isothiocyanate derivative of norlevorphanol. (B) Displacement of bound ^{3}H-labeled ligands from rat brain membranes by etonitazene at the concentrations shown. The ^{3}H-labeled ligands used were DALAMID, 2 n*M* (△); [D-Ala^2,D-Leu^5]enkephalin, 2 n*M* (●); and morphine, 5 n*M* (○). Bound and free ligand were separated by gel filtration as described (*8*). (C) Competition between [^{3}H]DALAMID and unlabeled etonitazene for binding to rat brain membranes that had been incubated at 1 mg of protein per milliliter of solution in the absence of opiate (○) or in the presence of 20 n*M* FIT (●), 20 n*M* BIT (▲), or 50 n*M* FAO (△), for 30 minutes at 37°C in 10 m*M* KPO_4 buffer, *p*H 8.0. The membranes were diluted, centrifuged, and suspended as described above. Portions were incubated for 10 minutes at 37°C in the presence of 8 n*M* [^{3}H]DALAMID and the indicated concentrations of etonitazene. Radioactive bound ligand was assayed after passage of the membrane suspension through Sephadex G-25 columns (*8*). The membranes used were prepared as described (*10*), and portions were stored at −70°C.

observations made with ^{3}H-labeled FIT suggest that a single molecular weight class of protein is specifically labeled in NG108-15 membranes (*11*).

KENNER C. RICE
ARTHUR E. JACOBSON
TERRENCE R. BURKE, JR.
BALBIR S. BAJWA
Laboratory of Chemistry, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, Bethesda, Maryland 20205
RICHARD A. STREATY
WERNER A. KLEE
Laboratory of General and Comparative Biochemistry, National Institute of Mental Health

References and Notes

1. J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, H. R. Morris, *Nature (London)* **258**, 577 (1975); A. F. Bradbury, D. G. Smyth, C. R. Snell, *Biochem. Biophys. Res. Commun.* **69**, 950 (1976); R. Guillemin, N. Ling, R. Burgus, *C. R. Acad. Sci. Ser. C* **282**, 783 (1976); C. H. Li and D. Chung, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1145 (1976); A. Goldstein, S. Tachibara, L. I. Lowney, M. Hunkapiller, L. Hood, *ibid.* **76**, 6666 (1979); K. Kangawa, H. Matsuo, M. Igarashi, *Biochem. Biophys. Res. Commun.* **86**, 153 (1979).
2. W. R. Martin, *Pharmacol. Rev.* **19**, 463 (1967); J. A. H. Lord, A. A. Waterfield, J. Hughes, H. W. Kosterlitz, *Nature (London)* **267**, 495 (1977); L. E. Robson and H. W. Kosterlitz, *Proc. R. Soc. London Ser. B* **205**, 425 (1979); J. R. Smith and E. J. Simon, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 281 (1980); H. W. Kosterlitz, J. A. H. Lord, S. J. Paterson, A. A. Waterfield, *Br. J. Pharmacol.* **68**, 333 (1980); R. Schulz, M. Wuster, H. Krenss, A. Herz, *Mol. Pharmacol.* **18**, 395 (1980).
3. A. Pfeiffer and A. Herz, *Biochem. Biophys. Res. Commun.* **101**, 38 (1981).
4. T. T. Lee, R. E. Williams, C. F. Fox, *J. Biol. Chem.* **254**, 11787 (1979); E. Hazum, K.-J. Chang, Y. Shechter, S. Wilkinson, P. Cuatrecasas, *Biochem. Biophys. Res. Commun.* **88**, 841 (1979); M. Smolarsky and D. E. Koshland, Jr., *J. Biol. Chem.* **255**, 7244 (1980); R. E. Venn and E. A. Barnard, *ibid.* **256**, 1529 (1981); C. Zioudrou, D. Varoucha, S. Loukas, R. A. Streaty, W. A. Klee, *Life Sci.* **31**, 1671 (1982).
5. A. Goldstein and R. Schulz, *Life Sci.* **16**, 1843 (1975); P. S. Portoghese, D. L. Larson, J. B. Jiang, A. E. Takemori, T. P. Caruso, *J. Med. Chem.* **21**, 598 (1978); T. P. Caruso, A. E. Takemori, D. L. Larson, P. S. Portoghese, *Science* **204**, 316 (1979); P. S. Protoghese, D. L. Larson, L. M. Sayre, D. S. Fries, A. E. Takemori, *J. Med. Chem.* **23**, 233 (1980); G. W. Pasternak and E. F. Hahn, *ibid.*, p. 674.
6. J.-K. Chang and P. Cuatrecasas, *J. Biol. Chem.* **254**, 2610 (1979).
7. M. G. C. Gillan, H. W. Kosterlitz, S. J. Paterson, *Br. J. Pharmacol.* **70**, 481 (1980).
8. W. F. Simonds, G. Koski, R. A. Streaty, L. M. Hjelmeland, W. A. Klee, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4623 (1980).
9. W. A. Klee and M. Nirenberg, *ibid.* **71**, 3474 (1974).
10. W. A. Klee and R. A. Streaty, *Nature (London)* **248**, 61 (1974).
11. W. A. Klee, W. F. Simonds, F. W. Sweat, T. R. Burke, Jr., A. E. Jacobson, K. C. Rice, *FEBS Lett.* **150**, 125 (1982).

5 August 1982; revised 28 September 1982

Toxic Shock Syndrome and Lysogeny in *Staphylococcus aureus*

Abstract. *Lysogeny, or the presence of temperate bacteriophage, was demonstrated, by means of two* Staphylococcus aureus *indicator strains, in 11 of 12 strains of* S. aureus *isolated from patients with toxic shock syndrome. Only 1 of 18 strains of* S. aureus *that were not associated with toxic shock syndrome showed the presence of bacteriophage. A laboratory strain of* S. aureus *was lysogenized by bacteriophage from two of the toxic shock–associated strains. These results add support to the theory that lysogeny by one or more bacteriophage in certain strains of* S. aureus *may be responsible for the pathogenesis of toxic shock syndrome.*

The toxic shock syndrome (TSS) is associated with *Staphylococcus aureus* and is characterized by fever, hypotension, desquamating rash, as well as other symptoms (*1, 2*). The syndrome has occurred in menstruating and nonmenstruating women, men, and children and has been associated with tampon use at menstruation, as well as staphylococcal empyema, septic abortions, fasciitis, osteomyelitis, and abscesses (*3, 4*). Although the syndrome may have occurred in the past (*5*), an increased incidence of the disease particularly in menstruating young women has only been noticed in the past 4 years.

According to the work of several investigators, the various symptoms and signs of TSS could be explained by the actions of a toxin. Two toxins associated with the extracellular products of *S. aureus* in TSS are enterotoxin F (*6*) and pyrogenic exotoxin C (*7*). Pyrogenic exotoxin C is believed to be a form of enterotoxin F (*8*). Pyrogenic exotoxin C is characterized by an isoelectric point of 7.2, a molecular weight of 22,000, pyrogenicity, enhancement of the effects of endotoxin, enhancement of a positive Dick test, suppression of immunoglobulin M synthesis after a second antigenic exposure, and ability to stimulate T cell proliferation (*8*).

Many of the signs and symptoms of TSS, such as fever, rash, and shock, are similar to those of scarlet fever in which the erythrogenic toxin of *Streptococcus pyogenes* plays a significant role. The production of the exotoxin of *S. pyogenes* (*9*) isolated in scarlet fever as well as the diphtheria toxin of *Corynebacterium diphtheriae* (*10*) have been clearly shown to be the result of lysogenic conversion. This term is used to describe a process by which genetic material of the bacteriophage is incorporated into the DNA of the bacterium, thereby conferring certain characteristics on the host strain. This is in contrast to virulent bacteriophage infection which results in complete lysis of the bacterium and release of viral progeny. The process of lysogenic conversion has been most extensively studied in the *Corynebacterium* in which a bacteriophage carrying the toxin gene is able convert a non-toxin–producing bacterium to a toxin producer [for a review, see (*11*)].

On the basis of these observations, we postulated that the toxin (or toxins) responsible for TSS may be under the same genetic control. In this report we present evidence that a significant number of TSS-associated *S. aureus* strains are lysogenized by one or more particular bacteriophage that are not found in non-TSS–associated strains, and that bacteriophage derived from the TSS-associated strains can be used to lysogenize another strain of *S. aureus*.

Strains of *S. aureus* associated with cases of TSS meeting the Centers for Disease Control (CDC) criteria for the disease were obtained from (i) CDC, Atlanta, Georgia, (ii) isolates from patients seen by us, (iii) New York Hospital–Cornell Medical Center, and (iv) samples sent by J. A. Giron and S. E. Read (Table 1). Non-TSS–associated strains, including vaginal isolates from asymptomatic women, were provided by some of the same sources. The *S. aureus* indicator strains 450, 450N (novobiocin-resistant), and 1830 were provided by S. Schaefler of the Public Health Research Institute of the City of New York.

Todd-Hewitt broth (*12*) was used to grow the organisms. Medium for agar plates (1 liter) consisted of 3 g of Casamino acids (Difco, Technical), 3 g of yeast extract (Difco), 5.9 g of NaCl, 20 ml of 2.5*M* Trizma base–HCl, *p*H 7.8, and 15 g of agar, to which 5 ml of 0.8*M* $CaCl_2$ was added just before the plates were poured. After the plates had solidified, excess moisture was removed from the agar surface as described by Zabriskie (*9*).

Screening for the presence of phage in each staphylococcal strain was achieved by growing an overnight culture (diluted 1:50) at 37°C to an optical density of 0.18 to 0.22 (measured at 650 nm in a Coleman 44 Spectrophotometer in a 16 by 125 mm tube). One milliliter of this suspension was used to flood the surface of agar plates. After the plates were tilted to remove excess fluid, they were allowed to stand at room temperature for 15 minutes. The plates were then inverted with the covers slightly off in a dry incubator at 37°C for 30 to 60 minutes. Plates containing these bacterial lawns