The Tryptophan Gene Cluster of *Staphylococcus aureus*

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SUMMARY

Tryptophan auxotrophs were isolated from *Staphylococcus aureus* strain 655 following mutagenesis with ultraviolet radiation, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or ethyl methanesulphonate. The mutants were placed in five mutant classes on the basis of auxotrophic response to and accumulations of tryptophan intermediates or derivatives. The tryptophan loci were mapped by comparing relative distances of each locus from two reference loci, *trpB* and *trpE*. Relative distances of loci were determined by ratio test analysis of cotransduction data. The tryptophan loci were shown to be contained on a cluster on the *S. aureus* genome; the mapping data suggested the gene order: *trpE*, *trp(DF)*, *trpC*, *trp(DF)*, *trpB*, *trpA*. The genetically distinct *trp(DF)* regions presumably control two enzymic functions in the conversion of anthranilic acid to 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate.

INTRODUCTION

The loci governing tryptophan biosynthesis are clustered and have similar arrangements in several members of the order Eubacteriales: *Escherichia coli* (Yanofsky & Lennox, 1959; Smith, 1967), *Salmonella typhimurium* (Blume & Balbinder, 1966); and *Bacillus subtilis* (Carlton & Whitt, 1969). The tryptophan loci of *Pseudomonas putida* (Gunsalus *et al.* 1968) are located in three separate linkage groups. In the more highly evolved fungi, the tryptophan loci are unlinked (Bonner, DeMoss & Mills, 1965; Mortimer & Hawthorne, 1966; Hunter & DeMoss, 1967; Roberts, 1967). These observations suggest that loss of clustering may be associated with evolutionary advancement. The conservation of genetic organization of tryptophan loci in species of the Eubacteriales lead us to surmise that tryptophan loci might be similarly arranged in other members of this order, especially those sharing a close evolutionary origin. The finding of a dissimilar arrangement would, following this hypothesis, constitute strong evidence of an evolutionary separation of the organisms considered. We have used this hypothesis to provide a comparison of the relatedness of two members of the order Eubacteriales, *Staphylococcus aureus* and *Micrococcus luteus*. These organisms are both members of the family Micrococcaceae, and have been the subject of much taxonomic debate (Baird-Parker, 1963). Though morphological similarities indicated a close relationship, modern taxonomic techniques have provided evidence of great dissimilarity in these organisms (Baird-Parker, 1963; Evans, 1965; Rosypal, Rosypalova & Horejs, 1966).

A study of the tryptophan loci of *Micrococcus luteus* was recently completed (Kloos & Rose, 1970). A previous study of the tryptophan loci in *Staphylococcus aureus* by Ritz & Baldwin (1962) showed linkage of ten tryptophan auxotrophs; however, their

mutants were not characterized as to the specific genetic loci involved. The objective of the present study was to elucidate the genetic organization of the tryptophan loci in *S. aureus*.

METHODS

Bacterial strains. *Staphylococcus aureus* strain 655 was the source of all tryptophan auxotrophs used in this study. *Salmonella typhimurium* strain 1T-2 tryptophan auxotrophs, *trpC3*, *trpD10*, *trpB14* and *trpE268*, were supplied by Dr E. Balbinder (Blume & Balbinder, 1966).

Media. Dehydrated media, Brain Heart Infusion (BHI) agar and Trypticase Soy Agar (TSA), were obtained from Difco. P agar and P and D broth were prepared as described by Naylor & Burgi (1956) and Pattee & Baldwin (1961), respectively. The defined medium (S broth), used for growth of *Staphylococcus aureus* mutants, was a modification (omission of tryptophan) of that described by Kloos & Pattee (1965a). The S agar was prepared by the addition of 1.5% (w/v) Special Noble agar (Difco) to S broth. The following modifications of S broth were used: ST broth (20 µg. tryptophan/ml.); SI broth (20 µg. indole/ml.; and SA broth (20 µg. anthranilic acid/ml.) A second medium, the glucose salts medium of Davis & Demerec as described by Lederberg (1950), was used for liquid culture of *Salmonella typhimurium* mutants.

Growth conditions. All cultures were grown at 37°, and liquid cultures were shaken either on a Burrell Wrist-Action shaker with cultures mounted over water baths, or on a rotary shaker (Fermentation Design, Inc., Allentown, Pennsylvania) housed in a 37° incubator.

Stock cultures of *Staphylococcus aureus* were maintained on BHI agar slopes in screw-capped tubes, and subcultures were maintained in cotton wool-plugged tubes. Culture transfers were incubated at 37° for 12 h. and stored at 4°. All experiments were made with 12- or 18-h. BHI agar slope cultures transferred from the subcultures. *Salmonella typhimurium* cultures were maintained in an identical manner on P agar slopes in cotton wool-plugged tubes.

Mutant isolation. Tryptophan auxotrophs were collected following mutagenesis of *Staphylococcus aureus* in four separate experiments using the following protocol: (1) bacteria from a 12 h. BHI slope culture were suspended in 5 ml. of either 0.85% (w/v) NaCl or sodium citrate; (2) mutagenesis by (a) irradiation (90 sec.) of bacterial suspension in an open agitated Petri dish at a distance of 39 cm. from a 'Mineralight Ultraviolet Lamp', Short Wave UVS-11 (115 V, 0.12 A) (Ultraviolet Products, Inc., San Gabriel, California); (b) incubation of bacteria in sodium citrate + 50 or 100 µg./ml. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; K and K Laboratories, Inc., Plainview, New York) for 1 h. at 37° (Adelberg, Mandel & Chen, 1965); (c) incubation of bacteria in 0.1 M-potassium phosphate buffer pH 7.0 + 0.4 M-ethyl methanesulphonate (EMS; Eastman Organic Chemicals, Rochester, New York) for 20 min. at 35° (Loveless & Howarth, 1959); (3) the treated bacteria were washed free from chemical mutagens and suspended in 5 ml. of P and D broth; (4) the broth suspension was shaken at a setting of three (Burrell Wrist-Action Shaker) for 5 h. at 37°, the organisms washed once in saline, and suspended in 10 ml. of S broth containing penicillin G (1 unit/ml.; Calbiochem, Los Angeles, California; Davis, 1949; Lederberg, 1950); (5) the culture was grown in the penicillin-containing broth for 26 h., the organisms washed, suspended in P and D broth, and stored at 4° while the titre of viable bacteria

was determined (growth in penicillin-broth was omitted from the experiment involving 50 µg. NTG/ml.); (6) samples of the stored bacterial suspension (diluted to produce 250 colonies per plate) were plated on 100 TSA plates; (7) mutant clones were selected following replica plating (Lederberg & Lederberg, 1952) to S agar and ST agar plates; (8) suspected mutants were streaked for isolation on TSA plates.

Mutant characterization and designation. All suspected mutant cultures were tested for response to tryptophan. Approximately 10^8 bacteria from 18 h. BHI agar slope cultures were spread on S agar plates, and a few crystals of tryptophan added at one side of each plate. Evidence of auxotrophic response to tryptophan was recorded after incubation for 48 h. The growth response of each tryptophan auxotroph to anthranilic acid and indole was determined by the same auxanographic technique.

To gain information about the tryptophan biosynthetic pathway of *Staphylococcus aureus*, and to characterize the biosynthetic block present in each mutant, de-repressed liquid cultures were examined for accumulations of known tryptophan biosynthetic intermediates and dephosphorylated derivatives. S broth supplemented with 2 µg. tryptophan/ml. (100 ml. in a 250 ml. Erlenmeyer flask) was inoculated to about 10^7 bacterial/ml. with bacteria from 18 h. ST broth cultures of each mutant strain. These cultures were incubated for 4 days with rotary shaking (250 rev./min.) at 37°. Bacteria were removed by centrifugation and the culture supernatant fluids acidified to pH 5.0 with 2 N-HCl. Tryptophan biosynthetic intermediates were extracted from the acidified supernatant fluids by shaking for 1 h. with an equal volume of ethyl acetate. The ethyl acetate extract was decanted and reduced to a volume of 0.3 ml. by forced aeration. The concentrated extracts were chromatographed on Whatman no. 1 filter paper by development with methanol + ethyl acetate + water (1 + 2 + 1, by vol.) 0.005 N in NH₄OH (Yanofsky, 1956). The chromatograms were dried at room temperature, and examined under short-wave ultraviolet radiation for the fluorescent compounds indole-3-glycerol (InG), anthranilic acid (AA) and 1-(o-carboxyphenyl-amino)-1-deoxyribulose (CDR). Further identification of AA and CDR spots was made by spraying separate chromatograms with Ehrlich reagent and triphenyl-tetrazolium (Yanofsky, 1960). Identification of indole and indole-3-glycerol in culture supernatant fluids was also made by the colorimetric techniques of Yanofsky (1955, 1956). The accumulations from previously identified mutants of *Salmonella typhimurium* (Blume & Balbinder, 1966) were used as standards for AA, CDR and InG. These mutants were grown in the glucose salts medium of Davis & Demerec (Lederberg, 1950) supplemented with 2 µg. tryptophan/ml.

Genetic locus designations were assigned to each mutant on the basis of auxotrophic tests and accumulation patterns as outlined in Table 1. The enzymic function presumed to be associated with each locus is shown in Fig. 1.

Phage preparation. All transduction studies were made with phage 83 of the International Typing Series. Phage 83 was propagated and titrated on various mutant strains by a modification (Pattee & Baldwin, 1961) of the agar-overlayer technique of Swanstrom & Adams (1951). Filter sterilized lysates were stored at 4° for periods up to 6 months.

Transduction techniques. All transduction experiments were made as described by Kloos & Pattee (1965*b*) with the exception that various dilutions of the transduction mixtures were plated in accordance with the observed transducibility of the recipient strain used.

The ratio test of Hartman, Loper & Serman (1960) was used to test linkage of the tryptophan loci, and to determine the relative distance of markers from a nutritionally distinct locus within the cluster. Cotransduction data are used to determine relative distances of loci. The relative distance is expressed as P , the ratio of prototrophs to 'total' colonies (donor-type and prototrophic transductants). This ratio, P , corresponds to the probability of independent integration of the donor-marker into the recipient. The ratio test relies on the ability to distinguish prototrophic transductants from donor-type cotransductants. Prototrophic recombinants and donor-type recombinants form distinctly different colonies, large and small, respectively, on S agar plates; the donor-types are presumably fed by the recipient organisms on the plate. The number of donor-type cotransductants could also be determined by subtracting the number of prototrophs on S agar plates from the number of colonies (prototrophs and donor-types indistinguishable) on supplemented agar plates. Two such types of transduction schemes are possible with tryptophan mutants: crosses involving *trpB* recipients (growth on tryptophan only; accumulation of indole) with all other mutant classes (growing on indole) as donors; and crosses involving *trpE* mutants (growing on AA) as donors with all other mutant classes (not growing on AA; accumulating AA) as recipients. These two transduction schemes were used to determine the relative distance of all mutant classes to the *trpB* locus, and relative to the *trpE* locus. By determining the relative distance of each locus from two different loci, the order of the individual loci should be determined.

Transduced bacteria from each cross were spread on 10 S agar plates to determine the numbers of both prototrophic and donor-type recombinants. In addition, transductants were spread on four supplemented agar plates: SI agar for crosses with *trpB* recipients; and SA agar for crosses with *trpE* donors. Colonies were scored following incubation for 72 h. The value of P was calculated from the total data from all 10 S agar plates for each cross. The data for 'total' colonies from the supplemented agar plates were used only when obvious discrepancies existed between the two sets of 'total' count data. This was observed when the donor-type colonies were poorly fed by the recipient background.

RESULTS

Mutant isolation and characterization. Two hundred and twenty-three tryptophan auxotrophs were isolated. Mutants *trp-1* through to *trp-16* were induced with ultra-violet radiation; mutants *trp-17* to *trp-203* with NTG; and mutants *trp-203* to *trp-219* with EMS. Mutants *trp-220*, -221, -222, -223 were supplied as uncharacterized tryptophan mutants by Dr P. A. Pattee (Iowa State University), and represent mutants *trp-17*, -20, -25, -26, respectively, of Dr Pattee's collection. Many of the mutants collected in this study had high reversion rates, were leaky, or had other growth abnormalities making them unsuitable for genetic analysis. These mutants were not further characterized. The remaining mutants were characterized in regard to their growth response to and accumulation of tryptophan biosynthetic intermediates. However, some of these mutants were eliminated from the study since they were unable to propagate phage 83 and/or be transduced. Table 1 lists mutants used in mapping experiments with the results of the biochemical characterizations, and the assigned genetic designations for each mutant. The pathway for tryptophan biosynthesis in *Staphylococcus aureus* (Fig. 1) suggested by the accumulation patterns of the

mutant classes was not shown to differ from the pathway occurring in other bacteria.

Cotransduction analysis. Phage 83 propagated on *trpE* donors gave donor-type transductants which were easily distinguishable from prototrophic colonies on S agar

Fig. 1. The tryptophan biosynthetic pathway of *Staphylococcus aureus*. Genetic loci and presumed enzymic functions are indicated above each step in the pathway. The classes *trpD* and *trpF* were not distinguished by biochemical characterizations, and all mutants which would presumably fall into these two categories were designated as *trp(DF)*. PR transferase, anthranilate phosphoribosyl transferase; PRA isomerase, phosphoribosyl anthranilate isomerase; InGP synthase, indole-3-glycerol phosphate synthase; PRA, *N*-(5'-phosphoribosyl) anthranilic acid; CDRP, 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate.

Table 1. *Biochemical characterization and genetic designation of tryptophan auxotrophs of Staphylococcus aureus*

Genetic locus designation	Representative mutants used in mapping	Tryptophan intermediate producing auxotrophic response	Tryptophan intermediate accumulated in de-repressed cultures
<i>trpE</i>	78, 85, 125 210, 218, 219	Anthranilic acid, indole, and L-tryptophan	None
<i>trp(DF)</i>	3, 155, 170 212, 214, 215	Indole or L-tryptophan	Anthranilic acid
<i>trpC</i>	47, 205, 221	Indole or L-tryptophan	CDR* and anthranilic acid
<i>trpB</i>	26, 207, 223	L-Tryptophan	Indole, InG,† CDR, and anthranilic acid
<i>trpA</i>	35, 213, 216	Indole or L-tryptophan	InG, CDR, and anthranilic acid

* CDR, 1-(*o*-carboxyphenylamino)-1-deoxyribulose.

† InG, indole-3-glycerol.

plates. Donor-type colonies were smaller in diameter: 0.2 to 1 mm. for donors, and 1.5 to 2.5 mm. for prototrophs. In addition, pigment development was slower in donor-type colonies. Some of the morphologically distinguishable colonies were sub-cultured from crosses representing each locus and tested for the auxotrophic feeding pattern of the donor from that cross. These suspected donor-type cultures were also tested for failure to be transduced by the donor which produced them. Approximately 95% of the suspected donor types were confirmed. Cotransduction was observed

between all *trp* loci; indicating linkage of all *trp* loci in a single transducing fragment of the *Staphylococcus aureus* genome. Ratio test analysis data are presented in Table 2 for *trpE* donor crosses and in Table 3 for *trpB* recipient crosses. The relative distance of each mutant marker from the *trpE* locus and from the *trpB* locus is expressed as the probability of independent integration of *trpE* donor markers into recipient mutants, and as the probability of independent integration of different donor markers into *trpB* recipients. These data suggest the order: *trpE*, *trp(DF)*, *trpC*, *trp(DF)*, *trpB*, *trpA* (Fig. 2). The average relative distance of each *trp* mutant from three reference *trpE* and *trpB* mutant sites is shown in Fig. 2. No significant gaps were observed in the *P*

Table 2. Ratio-test analysis of cotransduction from *trpE* donor crosses*

Recipient strain	Donor strain								
	<i>trpE</i> 210			<i>trpE</i> 218†			<i>trpE</i> 219		
	Prototrophs	Total	<i>P</i> ‡	Prototrophs	Total	<i>P</i>	Prototrophs	Total	<i>P</i>
<i>trpA</i> 216	3080	4419	0.70	2419	3776	0.64	2646	4019	0.65
<i>trpA</i> 213	2997	4853	0.62	1766	2931	0.60	1534	2738	0.56
<i>trpB</i> 223	948	1599	0.59	727	1181	0.61	2215	4313	0.51
<i>trpB</i> 207	2475	4402	0.56	2449	4352	0.56	2443	4959	0.49
<i>trpB</i> 26	2588	6284	0.41	3249	6852	0.47	1676	4419	0.38
<i>trp(DF)</i> 170	1184	3241	0.37	1146	2586	0.44	637	1930	0.33
<i>trpC</i> 221	926	2684	0.35	1075	2721	0.40	671	2240	0.30
<i>trpC</i> 47	—	—	—	51	235	0.22	—	—	—
<i>trpC</i> 205	611	2926	0.21	533	2772	0.19	284	1503	0.19
<i>trp(DF)</i> 212	1085	8282	0.13	548	6187	0.09	429	6340	0.07
<i>trp(DF)</i> 215	147	3759	0.05	299	2788	0.11	1550	11234	0.14

* The transduction mixture consisting of recipient bacteria (1.2×10^{10} /ml.) and phage at a multiplicity of infection of three was incubated with shaking for 30 min. at 37° and centrifuged. The transduced bacteria were resuspended, diluted (about 10^{-1} , varying with the recipient strain), and plated immediately.

† No cotransduction of the presumed unlinked markers, *hisG* 15 and *hisA* 1, was observed with *trpE* 218 and *trpA* 213.

‡ The ratio of prototrophs to total (prototrophs and donor-type) transductants.

Table 3. Ratio-test analysis of cotransduction data from *trpB* recipient crosses

Donor strain	Recipient strain								
	<i>trpB</i> 207			<i>trpB</i> 223			<i>trpB</i> 26		
	Prototrophs	Total	<i>P</i> *	Prototrophs	Total	<i>P</i>	Prototrophs	Total	<i>P</i>
<i>trpA</i> 216	224	3030	0.07	295	2375	0.12	33	296	0.11
<i>trpA</i> 213	246	3488	0.07	361	2785	0.13	29	284	0.10
<i>trpA</i> 35	169	3755	0.05	237	1707	0.14	48	261	0.18
<i>trp(DF)</i> 155	346	3810	0.09	79	687	0.11	58	321	0.18
<i>trp(DF)</i> 3	850	2292	0.37	317	2019	0.16	793	3455	0.23
<i>trp(DF)</i> 170	1162	3250	0.36	317	1766	0.18	—	—	—
<i>trpC</i> 221	1320	3183	0.41	533	2230	0.24	67	307	0.22
<i>trpC</i> 205	1533	3923	0.39	893	2885	0.31	2891	6280	0.46
<i>trpC</i> 47	676	1697	0.40	302	819	0.39	158	379	0.42
<i>trp(DF)</i> 214	2314	4063	0.50	910	2160	0.42	137	336	0.41
<i>trp(DF)</i> 215	1532	3555	0.43	733	1495	0.49	2559	5608	0.46
<i>trpE</i> 125	1933	3510	0.55	836	1663	0.50	2000	4259	0.46
<i>trpE</i> 78	2890	4698	0.62	1109	2263	0.49	3164	6233	0.51
<i>trpE</i> 85	1956	3146	0.62	691	1336	0.51	1934	3665	0.52

* The ratio of prototrophs to total (prototrophs and donor-type) transductants.

values from locus to locus. This is interpreted to mean that the tryptophan loci are contiguously clustered.

Abortive transduction analysis. We have been unable to find abortive transductants which would have been useful to identify mutant classes by complementation. The occurrence of tiny clones of stimulated mutant bacteria in the background growth of most mutant strains prevented the recognition of any such transductants. These clones occurred in controls with and without added phages at frequencies ten times that expected for abortive transductants; in general the ratio is about 5 to 20 abortive transductants to one complete transductant (Hayes, 1968). Mutant strains that were reisolated continued to exhibit this phenomenon.

Fig. 2. The order of tryptophan loci in *Staphylococcus aureus*. The average relative distance of each *trp* mutant site from three reference *trpE* (designated E) and *trpB* (designated B) mutant sites is shown in the top two maps. The general map of the tryptophan cluster is diagrammed below.

DISCUSSION

The proposed map order of tryptophan loci is based on two-point mapping data and is therefore somewhat tentative. However, the ratio-test analysis used provides internal control against the ambiguity often associated with two-point mapping (Hartman *et al.* 1960). Confirmation of this map order by deletion and/or three-point mapping would be desirable.

The apparent order of loci in the tryptophan gene cluster of *Staphylococcus aureus* is very similar to that of *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtilis*. This is yet another example of the similarity of this cluster in diverse members of the Eubacteriales. The gene order does not appear to be identical to that in *S. typhimurium*, for in this organism the tryptophan synthase B (*trpD*) and the indole-3-glycerol phosphate synthase (*trpE*) functions are adjacent (Blume & Balbinder, 1966). The order in *S. aureus* may be identical to that in the *B. subtilis* and *E. coli* clusters where the phosphoribosyl anthranilate isomerase (*trpF* and *trpC*, respectively) function maps between the *trpB* locus and the indole glycerol phosphate synthase function (Smith, 1967;

Carlton & Whitt, 1969). However, the two *trp*(*DF*) loci in the *S. aureus* cluster were not defined, and a different order, *trpE*, *trpF*, *trpC*, *trpD*, *trpB*, *trpA*, may be correct.

An exception to this conserved pattern of *trp* loci in the eubacteria has been found by Kloos & Rose (1970) in *Micrococcus luteus*. The order, *trpE*, *trpC*, *trpB*, *trpA*, common to all species of eubacteria examined, was retained; however, the *trp*(*DF*) function(s) were shown to be removed from the cluster. The results of the present study add significance to these findings.

Considering the similarity of the tryptophan gene clusters of organisms as diverse of *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*, the failure of all the tryptophan loci to cluster in *Micrococcus luteus* is significant. This provides evidence of genetic dissimilarity of *S. aureus* and *M. luteus*. These genetic data further support the separation of staphylococci and micrococci by physiological criteria (Evans, Bradford & Niven, 1955; Baird-Parker, 1963), and by DNA base composition (micrococci 66 to 73 % guanine+cytosine (GC), and staphylococci 30 to 36 % GC, Rosypal *et al.* 1964). It is hoped that additional studies on the organization of other functionally related loci will prove helpful in further defining the relationship of these organisms.

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