TRANSFORMATION MAPPING OF TRYPTOPHAN LOCI IN *MICROCOCCUS LUTEUS*¹

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THE recent demonstration of transformation in *Micrococcus luteus* strain lysodeikticus (formerly *Micrococcus lysodeikticus*) has made possible genetic analysis of this species (KLOOS 1968; MAHLER and GROSSMAN 1968; OKUBO and NAKAYAMA 1968; KLOOS and SCHULTES 1969). Using optimum conditions, transformation frequencies on the order of 0.1–0.7 percent of colony-forming units have been obtained with adenine and tryptophan auxotrophs (KLOOS 1969a; ROSE and KLOOS 1969). These levels, although not as high as have been produced in some other organisms, are adequate for the initiation of genetic studies.

This investigation is concerned with evaluating transformation as a tool in mapping the tryptophan loci of the micrococcal genome. In addition, some information on tryptophan biosynthesis in *M. luteus* is obtained from growth responses of tryptophan auxotrophs on tryptophan precursors and from the accumulation patterns of mutant cultures.

MATERIALS AND METHODS

Bacterial strains: *Micrococcus luteus* tryptophan auxotrophs were derived from an adenine-independent mutant (*ade*⁺*-1*) of the high transforming strain lysodeikticus ISU (formerly *M. lysodeikticus* strain ISU) (KLOOS and SCHULTES 1969) and *M. luteus* strain lutea ATCC 272 (formerly *Sarcina lutea* strain ATCC 272). Mutant auxotrophs were induced by treatment of the parent strain with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (300 µg/ml) (ADELBERG, MANDEL and CHEN 1965) or with ethyl methanesulphonate (EMS) (LOVELESS and HOWARTH 1959).

The tryptophan auxotrophs *trpC3*, *trpD10*, *trpB14*, and *trpE268* of *Salmonella typhimurium* strain LT-2 were used as sources of tryptophan intermediates for references in paper chromatography. They were obtained from Dr. E. BALBINDER (Syracuse University, Syracuse, New York) and have been described by BLUME and BALBINDER (1966).

Media: The complex broth (P broth) and agar (P agar) used to propagate *M. luteus* strains have been described by NAYLOR and BURGI (1956). The defined broth and agar media have been described by KLOOS (1969a) and KLOOS and SCHULTES (1969), respectively. Defined agar media were supplemented with 0.5% casein acid hydrolysate when used in the detection of donor-type colonies in cotransformation studies. *S. typhimurium* strains were grown in the glucose-salts broth of DAVIS and DEMEREC described by LEDERBERG (1950).

Biochemical characterization of tryptophan auxotrophs: Tryptophan auxotrophs of *M. luteus* strains were initially tested for their growth response to anthranilic acid, indole, and L-tryptophan. About 10⁷ cocci of each mutant strain were spread over the surface of two defined agar plates.

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Several crystals of L-tryptophan and anthranilic acid were placed on opposite sides of one of the plates and indole was placed in the center of the other plate. Plates were incubated at 34°C for 48 hr and then scored for evidence of growth.

Tryptophan auxotrophs were further characterized according to their accumulation of tryptophan precursors and/or derivatives. Ascending paper chromatography was used to identify anthranilic acid, 1-(*o*-carboxyphenylamino)-1-deoxyribulose (CDR), and indole glycerol according to modifications of the techniques of DOY and GIBSON (1959), SMITH and YANOFSKY (1960), and YANOFSKY (1956). Cultures of each mutant and the parent strains were grown on a P agar slant for 18 hr and then washed twice in 2 ml saline. Aliquots of 0.3 ml (about 1×10^9 colony-forming units) were used to inoculate 250 ml Erlenmeyer flasks containing 30 ml defined broth supplemented with 2 $\mu\text{g/ml}$ L-tryptophan. Broth cultures were shaken for 12 hr (*M. luteus* strain lutea ATCC 272) or 24–36 hr (*M. luteus* strain lysodeikticus ISU) on a rotary shaker (Fermentation Design, Inc., Allentown, Pennsylvania) at 250 rpm at 34°C. *S. typhimurium* tryptophan auxotrophs were grown in the broth medium of DAVIS and DEMEREC supplemented with 2 $\mu\text{g/ml}$ L-tryptophan for 24 hr at 37°C. Cultures were centrifuged and the supernatant fluids were acidified to pH 5.2 with HCl. An equal volume of ethyl acetate was added to the acidified solution to extract tryptophan intermediates. Extraction was accomplished by shaking the mixture vigorously with a Burrell Wrist-Action Shaker (Burrell Corporation, Pittsburgh, Pennsylvania) for 1 hr at room temperature. The ethyl acetate was then decanted into a beaker and evaporated by forced air to 0.3 ml. The ethyl acetate extract of each culture was applied as a spot (0.03 ml) on Whatman No. 1 filter paper. Chromatograms were developed in a methanol : ethyl acetate : water (1:2:1) + 0.005N NH_4OH solvent system for 2 hr until the front was 15 cm from the origin. Chromatograms were dried in air at room temperature for 10 min and then illuminated with ultraviolet light from a Short Wave (UVS-11) Mineralight (Ultra-violet Products, Inc., San Gabriel, California) for the identification of intermediates. Separate chromatograms were also sprayed with Ehrlich's reagent and triphenyltetrazolium to distinguish anthranilic acid and CDR. Ethyl acetate extracts were also tested for the presence of indole by the method of YANOFSKY (1955) and for indole glycerol by the ferric chloride method of YANOFSKY (1956).

Preparation of transforming DNA: The culture of donor strains for DNA isolation and the preparation of transforming DNA have been described previously (KLOOS 1969b).

Procedure for transformation: An 18 hr P agar slant culture of the recipient strain was suspended in 1 ml saline and diluted 1/100 in saline. Aliquots of 0.1 ml (about 5×10^6 colony-forming units) from the diluted suspension were added to duplicate tubes containing 1 ml defined broth supplemented with 20 $\mu\text{g/ml}$ L-tryptophan. Mixtures were shaken in a 34°C water bath with a Burrell Wrist-Action Shaker at a setting of 4 for 24 hr. After growth, cocci (about $1-3 \times 10^8$ colony-forming units/ml) were centrifuged and resuspended in 1 ml transformation buffer: 0.05M Tris (hydroxymethyl) aminomethane + 0.01M SrCl_2 , pH 7.0. DNA, 10 μg for the construction of double *trp* mutants or 0.1 μg in crosses (saturation level is approximately 1 $\mu\text{g/ml}$), in 0.1 ml saline buffer was added to each tube and the mixtures were shaken in a 30°C water bath at a setting of 4 for 30 min. DNA uptake was terminated by the addition of deoxyribonuclease (5 $\mu\text{g/ml}$) and 0.005M MgSO_4 with incubation at 37°C for 15 min. After treatment, cocci were centrifuged and resuspended in 1 ml saline. Aliquots of 0.1 ml were taken from an appropriate saline dilution of the original suspension and spread on duplicate defined agar plates. Additional aliquots were spread on duplicate defined agar plates containing either anthranilic acid or indole (20 $\mu\text{g/ml}$) as indicated. Two separate transformations were prepared for each cross and the data presented in experiments are the average of the results. Prototrophs were scored after incubation at 34°C for 72 hr. Donor-type cotransformants were scored after incubation for 4 days.

Cotransformation analysis: Determination of linkage and an estimate of the relative distance of tryptophan loci from a reference marker (a phenotypically distinguishable tryptophan class, i.e., *trpE*: growth with anthranilic acid or *trpB*: no growth on indole) was accomplished using the ratio test (HARTMAN, LOPER and ŠERMAN 1960). The rationale of the ratio test in estimating relative marker distances is that when one auxotrophic marker is in the donor DNA and an-

other is in the recipient DNA the frequency of prototrophic transformants will be directly related to the distance separating mutant sites, whereas the frequency of donor-type cotransformants will be inversely related to distance. Donor-type cotransformants could be detected on defined agar supplemented with anthranilic acid (*trpE* donors) or indole (*trpE*, *C*, and *A* donors) and their numbers calculated by the difference in numbers of large prototrophic colonies on unsupplemented media. Donor-type cotransformants can also be readily recognized on unsupplemented defined agar media by their small size (0.6 mm) compared to the larger prototrophic transformants (3.0 mm) and paler color. Presumably, the donor-type cotransformants are being stimulated by compounds accumulated by the recipient background.

Donor-type cotransformants were verified by (1) growth on anthranilic acid (*trpE* donors), indole (*trpE*, *C*, or *A* donors) or L-tryptophan but no growth in their absence and (2) failure to undergo recombination as recipient with DNA from the original donor strain while still able to produce prototrophic transformants at normal frequencies with DNA from the *trp*⁺ strain lysodeikticus ISU (*ade*⁺-1).

Three-point crosses: Double mutants containing the reference marker *trpE16* were constructed by transforming a recipient strain *hisD61 trpE16* with DNA prepared from a donor strain carrying the desired additional tryptophan marker. In these crosses, histidine prototrophs were replica plated on defined agar supplemented with anthranilic acid. Marker *hisD61* is unlinked to *trpE16* and selection of *his*⁺ clones enriches for those cells that are competent to take up DNA. The presence of the donor tryptophan marker was established by failure of the histidine prototroph to grow on anthranilic acid agar. The double mutant nature of these strains was verified by (1) their failure to accumulate tryptophan intermediates and (2) failure to produce prototrophic recombinants when crossed with either parent strain and ability to produce prototrophic transformants with DNA from the wild-type strain lysodeikticus ISU (*ade*⁺-1). Three-point crosses were conducted according to the procedures of CARLTON (1967). The rationale for marker order assignment is that if the single tryptophan mutant site of a donor is located between the reference marker *trpE16* and a second tryptophan marker in the recipient, then it would require a quadruple crossover to yield a prototroph; however if the donor mutant site is outside, then only a double crossover would be required. Prototrophs are calculated as the percentage of the recombinant classes *trpE*+*X*+*Y*+ and *trpEX*+*Y*+ (*X* and *Y* represent the other two tryptophan markers in the cross). The *trpEX*+*Y*+ recombinant class was estimated by the difference in the number of colonies produced on defined media and defined media supplemented with anthranilic acid.

RESULTS

Biochemical characterization of tryptophan auxotrophs: A total of 72 tryptophan auxotrophs (*trp*-1 through *trp*-24; *trp*-25 through *trp*-47; *trp*-48 through *trp*-72) of *Micrococcus luteus* strain lysodeikticus ISU were isolated in three separate experiments using NTG as the inducing agent and 30 tryptophan auxotrophs (*trp*-73 through *trp*-102) were isolated in one experiment using EMS. Thirty-five tryptophan auxotrophs (*trp*-103 through *trp*-137) of *M. luteus* strain lutea ATCC 272 were isolated in one experiment using NTG. Sixty-nine of the mutants were omitted from further study due to leakiness, poor growth in defined broth, or too high a reversion rate. The remaining 68 mutants are listed in Table 1. All strain lysodeikticus ISU mutants, except *trpB32* and *trpB33*, were genetically recombinable and, therefore, of independent origin. Strain lutea ATCC 272 mutants cannot be tested for recombination in homologous crosses, as recipient competence has not been demonstrated in this strain. However, several of the mutants can be differentiated by other secondary characteristics. Mutant *trp(DF)105* can be distinguished from other *trp(DF)* mutants by its water-

TABLE 1

Biochemical characterization of Micrococcus luteus tryptophan auxotrophs

Mutant class	Representative strains	Growth response	Accumulated intermediates
<i>trpE</i>	lysodeikticus ISU: 16, 31, 54, 66, 71, 79, 82, 96, 102 lutea ATCC 272: 106, 111, 113, 114, 115, 118, 122, 124	anthranilic acid, indole or L-tryptophan	none
<i>trp(DF)</i>	lysodeikticus ISU: 2, 38, 43, 52, 63, 73, 98 lutea ATCC 272: 104, 105, 120	indole or L-tryptophan	anthranilic acid
<i>trpC</i>	lysodeikticus ISU: 13, 23, 29, 46 lutea ATCC 272: 107, 119, 121, 123	indole or L-tryptophan	anthranilic acid and CDR
<i>trpB</i>	lysodeikticus ISU: 1, 7, 10, 12, 18, 20, 24, 26, 30, 32, 33, 36, 51, 53, 56, 57, 58, 59, 65, 68, 70, 72, 81, 86, 88, 94	L-tryptophan	anthranilic acid, CDR, indole glycerol, and indole
<i>trpA</i>	lysodeikticus ISU: 6 lutea ATCC 272: 103, 108, 109, 110, 116, 117	indole or L-tryptophan	anthranilic acid, CDR, and indole glycerol

soluble pigment. Mutant *trpC107* grows considerably slower in defined broth than the other *trpC* mutants. Mutant *trpA103* is slightly leaky, mutants *trpA110* and *trpA117* have an orange fluorescent pigment, and mutant *trpA116* is pink pigmented.

Growth responses and accumulation patterns subdivided mutants into five classes (Table 1) similar to those of other bacteria. Mutants responding to anthranilic acid, indole, or L-tryptophan failed to accumulate detectable tryptophan intermediates. Since the defined medium contained both L-tyrosine and L-phenylalanine, both mutants blocked earlier than anthranilate synthetase (multiple aromatic auxotrophs) and anthranilate synthetase (*trpE*) auxotrophs would be included in this group (GIBSON and PITTARD 1968). Multiple aromatic auxotrophs were omitted from further study. They were identified by (1) their requirement for both L-tyrosine and L-tryptophan (growth of the parent strain was reduced by omitting L-tyrosine but not as severely), (2) when used as donors in crosses with suspected *trpE* recipients they produced a high frequency of prototrophic transformants which was similar to the frequency obtained when the donor was the parent strain, and (3) their failure to produce donor-type recombinants with recipient mutants in the other four classes. The latter two criteria would suggest the absence of linkage to tryptophan markers. *trpE* mutants, on the other hand,

FIGURE 1.—A genetic map of tryptophan loci and the presumed tryptophan biosynthetic pathway in *Micrococcus luteus*. Positions of mutant sites are designated by the small lines intersecting the long line that represents a portion of the genome. Mutants with uncertain intragenic positions are noted without the use of intersecting lines. Mutants *E66* and *E118* are placed on the map on the basis of growth-accumulations and cotransformation data, rather than three-point tests (as are the other mutants). The positions of *M. luteus* strain lysodeikticus ISU mutants are shown above the lines and those of strain *lutea* ATCC 272 are shown below. The functions of each gene are designated by the solid lines drawn to the specific reaction in the tryptophan pathway. It is uncertain from the biochemical data which of the two reactions or if both are represented by (*DF*) mutants.

did not require *L*-tyrosine and produced donor-type recombinants when crossed with *trpA*, *trpB*, and *trpC* mutants.

Mutants responding to indole or *L*-tryptophan were further characterized according to their accumulations. *trpA* mutants accumulated anthranilic acid, CDR, and indole glycerol, *trpC* mutants accumulated anthranilic acid and CDR, and *trp(DF)* mutants accumulated only anthranilic acid.

trpB mutants grew only in the presence of *L*-tryptophan and accumulated anthranilic acid, CDR, indole glycerol, and indole. The presumed *L*-tryptophan biosynthetic pathway is shown in Figure 1. Repression or feedback inhibition appears to affect the production of tryptophan intermediates. Accumulations resulted in intense fluorescent spots on chromatograms and intense color reactions when mutants were grown in the presence of 1–4 $\mu\text{g/ml}$ *L*-tryptophan. Intermediates were not observed if mutants were grown in the presence of *L*-tryptophan in excess of 4–20 $\mu\text{g/ml}$ (depending upon the particular mutant).

Cotransformation analysis: Evidence for linkage of *trpE*, *trpC*, *trpB*, and *trpA* loci was obtained by the appearance of donor-type cotransformants in crosses of representative mutants with *trpE* and *trpB* mutants (Tables 2 and 3). Donor-type cotransformants were not observed in crosses with *trp(DF)* mutants, used either as donors or recipients, thus suggesting a lack of linkage of this class to other

TABLE 2

*The probability of independent integration of trpE markers into different tryptophan mutants of Micrococcus luteus**

Recipient strain lysodeikticus ISU	Donor strain		lutea ATCC 272 <i>trpE118</i>
	lysodeikticus ISU <i>trpE16</i>	<i>trpE66</i>	
<i>trpA6</i>	0.38	0.29	0.30
<i>trpB7</i>	0.32	0.22	0.26
<i>trpB94</i>	0.30	0.19	0.22
<i>trpB18</i>	0.32	0.21	0.21
<i>trpC23</i>	0.28	0.19	0.18
<i>trpC46</i>	0.25	0.16	0.18
<i>trpC29</i>	0.22	0.13	0.18
<i>trpC13</i>	0.15	0.13	0.14

* Figures represent the probability of independent integration (p) which is equal to the number of prototrophic transformants divided by the number of prototrophic + donor-type transformants.

tryptophan loci. Mutants from both *M. luteus* strains, lysodeikticus ISU and lutea ATCC 272, were used in crosses to compare the arrangement of tryptophan loci in these organisms. Mutants from strain lutea ATCC 272 were used only as donors.

The relative distance of *trpA*, *trpB*, and *trpC* mutant sites from *trpE* mutant sites is demonstrated by the probability of independent integration (p) as shown in Table 2. The total number of colonies scored to estimate p was in a range of 1448–5970 in crosses using strain lysodeikticus ISU as donor and 246–1763 in crosses using strain lutea ATCC 272. Some ambiguity was observed in distances

TABLE 3

*The probability of independent integration of different tryptophan markers into trpB mutants of Micrococcus luteus**

Donor strain	Recipient strain lysodeikticus ISU		
	<i>trpB7</i>	<i>trpB94</i>	<i>trpB18</i>
lysodeikticus ISU:			
<i>trpE16</i>	0.32	0.30	0.32
<i>trpE66</i>	0.22	0.24	0.27
<i>trpC13</i>	0.22	0.18	0.30
<i>trpC29</i>	0.16	0.15	0.20
<i>trpC46</i>	0.10	0.17	0.17
<i>trpC23</i>	0.09	0.08	0.14
<i>trpA6</i>	0.12	0.15	0.16
lutea ATCC 272:			
<i>trpE118</i>	0.26	0.22	0.21
<i>trpC107</i>	0.23	0.17	0.22
<i>trpC121</i>	0.19	0.12	0.15
<i>trpA110</i>	0.09	0.06	0.10
<i>trpA116</i>	0.07	0.03	0.04

* Figures represent the probability of independent integration (p) which is equal to the number of prototrophic transformants divided by the number of prototrophic + donor-type transformants.

TABLE 4

*Two-point crosses between trp(DF) mutants and various trp mutants of the tryptophan cluster**

Donor strain	Recipient strain lysodeikticus ISU				
	<i>trpA6</i>	<i>trpC46</i>	<i>trp(DF)2</i>	<i>trp(DF)43</i>	<i>trp(DF)73</i>
lysodeikticus ISU:					
<i>trp</i> ⁺	1,092	2,060	781	54	364
<i>trpA6</i>	2	833	766	45	488
<i>trpB18</i>	84	524	658	53	349
<i>trpC46</i>	316	8	673	47	371
<i>trpC29</i>	352	188	680	47	410
<i>trpE16</i>	413	489	611	48	349
<i>trp(DF)2</i>	1,113	2,137	0	4	49
<i>trp(DF)38</i>	1,047	1,653	25	7	18
<i>trp(DF)43</i>	1,340	1,841	94	0	16
<i>trp(DF)52</i>	1,316	1,720	26	2	40
<i>trp(DF)63</i>	1,114	1,880	94	4	31
<i>trp(DF)73</i>	965	2,072	43	4	0
<i>trp(DF)98</i>	960	1,603	71	6	21
lutea ATCC 272:					
<i>trp</i> ⁺	461	840	582	31	212
<i>trpA116</i>	32	276	487	27	192
<i>trpC121</i>	106	12	490	22	173
<i>trp(DF)104</i>	489	846	12	2	4
<i>trp(DF)105</i>	535	931	17	1	10

* Figures represent the frequency of prototrophic transformants per 10⁶ colony-forming units.

between mutant sites; however, these results would suggest that the relative distance of tryptophan loci from the *trpE* locus increases in the order: *trpC*, *trpB*, *trpA*.

It is also possible to conduct another series of experiments involving cotransformation of tryptophan loci by using *trpE*, *trpC*, and *trpA* mutants as donors with *trpB* mutants as recipients (Table 3). The total number of colonies scored to estimate *p* was in a range of 724–2153 in crosses using strain lysodeikticus ISU as donor and 404–1650 in crosses using strain lutea ATCC 272. Results indicated that the relative distance of tryptophan loci from the *trpB* locus increases in the following order: *trpA* and *trpC* (approximately similar), *trpE*.

Each series of cotransformation experiments using first *trpE* and then *trpB* as the reference marker will not alone determine a map sequence of loci but only relative distances. However, data taken collectively from both series would imply that the sequence of tryptophan loci in *M. luteus* appears to be *trpE-trpC-trpB-trpA*. It would also appear that the arrangement of loci in the tryptophan regions in strains lysodeikticus ISU and lutea ATCC 272 is similar.

Further evidence of a distant separation of *trp(DF)* mutant sites from the main tryptophan cluster was obtained in two-point crosses between *trp(DF)* mutants and other tryptophan mutants (Table 4). Mutants of the tryptophan cluster produced significantly lower frequencies of prototrophic transformants when crossed to each other than when the parent strain was used as donor or when

crossed to *trp(DF)* mutants. Likewise, *trp(DF)* mutants produced low frequencies of prototrophic transformants when crossed to each other, but high frequencies when the parent strain was used as donor or when crossed to other tryptophan mutants.

Three-point transformation: The results of three-point transformation crosses of representative mutants of four tryptophan classes are shown in Table 5. CARLTON (1966) found that in mapping tryptophan mutant sites in *Bacillus subtilis* such crosses fell into two classes. Those assumed to require a quadruple crossover to produce prototrophic transformants had less than 25 percent prototrophs, and those assumed to require a double crossover had levels greater than 40 percent. As the data in Table 5 indicate, crosses between single tryptophan donor mutants and double tryptophan recipient mutants of *M. luteus* were comparable to those found in *B. subtilis*. The percentage of prototrophic transformants in crosses where the donor mutant site was assumed to be between the two recipient mutant sites was 32% or less, whereas in crosses where the donor mutant site was assumed to be outside these sites, it was 49% or greater. The total number of recombinants scored in intergenic crosses of strain lysodeikticus ISU was in a range of 446–1222; the total number of recombinants in intragenic crosses was 138–192. The number of recombinants scored in crosses between strain lutea ATCC 272 and strain lysodeikticus ISU was significantly lower, with a range of 42–180 in intergenic crosses and 0–98 in intragenic crosses.

TABLE 5

*Three-point transformation mapping of the tryptophan cluster of Micrococcus luteus**

Donor strain (Y ⁻)	<i>trpC13</i>	<i>trpC29</i>	Recipient strain lysodeikticus ISU (<i>trpE16X⁻</i>)				<i>trpB7</i>	<i>trpA6</i>
	<i>trpC46</i>	<i>trpC23</i>	<i>trpB18</i>	<i>trpB94</i>				
lysodeikticus ISU:								
<i>trp⁺</i>	91	87	81	87	80	78	78	72
<i>trpE16</i>	0	0	0	0	0	0	0	0
<i>trpC13</i>	0	15	20	20	18	19	19	13
<i>trpC29</i>	55	0	32	17	16	17	15	10
<i>trpC46</i>	68	57	0	21	26	20	14	18
<i>trpC23</i>	67	73	61	0	25	18	25	19
<i>trpB18</i>	75	72	78	79	0	20	22	11
<i>trpB94</i>	80	68	83	79	49	0	30	17
<i>trpB7</i>	74	88	68	63	67	76	0	21
<i>trpA6</i>	88	86	75	90	83	92	85	0
lutea ATCC 272:								
<i>trpC121</i>	64	NR	13	19	13	13	14	11
<i>trpC107</i>	NR	20	17	14	12	10	15	10
<i>trpA103</i>	—	—	—	92	96	—	75	10
<i>trpA108</i>	—	—	—	87	90	—	86	9
<i>trpA110</i>	—	—	—	91	86	—	85	10
<i>trpA116</i>	—	—	—	78	75	—	72	12

* Figures represent the percentage of *trpE⁺* recombinants in the *X+Y⁺* recombinant classes. The notation — = crosses that were not performed and NR = crosses failing to produce recombinants.

The sequence of tryptophan mutant sites in the tryptophan cluster of *M. luteus* strain lysodeikticus ISU, determined by three-point transformation analysis, appears to be *trpE16-trpC13-trpC29-trpC46-trpC23-trpB18-trpB94-trpB7-trpA6*. Mutant sites *trpA103*, *trpA108*, *trpA110*, and *trpA116* of strain lutea ATCC 272 map between *trpB7* and *trpA6* sites of strain lysodeikticus ISU. Mutant site *trpC121*, of this strain, is located between *trpC13* and *trpC46*; *trpC107* is in a region between *trpE16* and *trpC29*.

All of the *trp(DF)* mutants of both *M. luteus* strains produced high levels of prototrophic transformants, 76% or greater, when crossed with the various double mutant recipients. This would imply that these mutants map outside of the *trpE16-trpA6* region and is consistent with other information suggesting a lack of linkage to the tryptophan cluster. When the various tryptophan mutants and wild-type strain were crossed to a double mutant recipient *trpE16(DF)73*, prototrophic recombinants were recovered in only some crosses, at 0.2% or less, and were probably double transformants. These results suggest the possibility that mutant sites *trpE16* and *trp(DF)73* are located on different DNA fragments.

Reciprocal crosses were conducted with strain lysodeikticus ISU in which the donor strain was a double mutant (in each case *trpE16* of lysodeikticus was present) and the recipient strain a single mutant. In each of these crosses, a double crossover would be required to yield a prototroph. If the single mutant site in the recipient is located outside of the two donor markers, then the percentage of prototrophic transformants would be high; in these experiments it was 74% or greater. On the other hand, if the single mutant site is located between the two donor markers, the frequency of prototrophic transformants would depend upon the distance of the recipient marker from the *trpE16* donor marker. In effect, this situation would be analogous to the cotransformation analysis described in the previous section, using *trpE* mutants as donors. Small donor-type cotransformants were detected on defined agar media in these crosses. The range of percentages of prototrophic transformants was 24% for recipient *trpC13* to 48% for recipient *trpB7*. The sequence of mutant sites reflected in the reciprocal crosses was consistent with the data from cotransformation studies and three-point transformation mapping using the reverse relationship of donor and recipient markers.

On the basis of both cotransformation and three-point transformation studies, the mutant sites affecting tryptophan biosynthesis can be represented in a genetic map as shown in Figure 1.

DISCUSSION

These studies have demonstrated the feasibility of transformation mapping in ordering tryptophan loci in *Micrococcus luteus*. Both cotransformation analysis and three-point transformation determined identical sequences of loci. Three-point transformation also allowed determination of the order of mutant sites within the three loci tested. Tryptophan auxotrophs mapped in four distinct and presumably contiguous regions (*trpE*, *trpC*, *trpB*, *trpA*) of the tryptophan cluster and in an unlinked gene or genes, *trp(DF)*.

Cotransformation of tryptophan loci in crosses between donor strain lutea

ATCC 272 and various tryptophan auxotrophs of strain lysodeikticus ISU and the positioning of mutant sites in three-point crosses between these organisms implies close genetic homology and an identical arrangement of tryptophan loci. Some evidence of a small, but yet significant, divergence in these organisms may be exemplified by the slightly lower frequency of transformation in crosses between strain lutea ATCC 272 and strain lysodeikticus ISU than found in homologous crosses. The above findings are consistent with proposals to include both strains, which were originally designated in separate genera, *Micrococcus* and *Sarcina*, as members of the species *M. luteus* (BAIRD-PARKER 1965; KLOOS 1969b; KOCUR and MARTINEC 1962; ROSYPAL, ROSYPALOVA and HOREJS 1966).

Tryptophan loci in the eubacteria *Escherichia coli* (YANOFSKY and LENNOX 1959), *Salmonella typhimurium* (BLUME and BALBINDER 1966), *Bacillus subtilis* (CARLTON and WHITT 1969), and *Staphylococcus aureus* (PROCTOR and KLOOS 1969) are clustered into one region and appear to be arranged in a similar sequence. On the other hand, tryptophan loci are located in three linkage groups in the pseudomonad *Pseudomonas putida* (GUNSALUS *et al.* 1968). By comparison, *M. luteus* appears to have a tryptophan cluster composed of at least four loci, in a sequence similar to that found in other eubacteria studied, and a separate linkage group composed of at least one and possibly two loci. Enzyme studies are being pursued to further clarify gene functions. This is particularly necessary to resolve *D* and/or *F* genes, indistinguishable from one another by mutant growth stimulation or accumulation patterns.

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SUMMARY

Tryptophan auxotrophic mutants of *Micrococcus luteus* strain lysodeikticus ISU (formerly *Micrococcus lysodeikticus* strain ISU) and mutants of *M. luteus* strain lutea ATCC 272 (formerly *Sarcina lutea* strain ATCC 272) were placed into five phenotypic classes according to their growth responses and by their accumulation of intermediates. The pathway of tryptophan biosynthesis in *M. luteus* appears to be similar to that found in other eubacteria.—Cotransformation analysis and three-point transformation mapping suggest that four tryptophan loci are linked in a cluster and are in the sequence *trpE-trpC-trpB-trpA*. One other class of mutants, *trp(DF)*, presumed to be blocked in the conversion of anthranilic acid to 1-(*o*-carboxyphenylamino)-1-deoxyribulose, is not linked to this cluster. Three-point transformation tests allowed unambiguous mapping of tryptophan mutant sites within the cluster of four loci. Tryptophan loci in *M. luteus* strain lysodeikticus ISU and *M. luteus* strain lutea ATCC 272 show a high degree of genetic homology but some interstrain differences were noted.

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