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Biochemical and Genetic Analysis of Isoleucine and Valine Biosynthesis in *Staphylococcus aureus*

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After a prototrophic strain of *Staphylococcus aureus* had been exposed to diethyl sulfate, 28 isoleucine- and isoleucine-valine-dependent mutants (*ilv* mutants) were isolated. On the basis of auxanography, their ability to accumulate intermediates of isoleucine and valine biosynthesis, and intergeneric syntrophism with *ilv* mutants of *Salmonella typhimurium*, all mutants were placed into four groups, each of which corresponded to a presumed enzymatic deficiency, as follows: group A, deficient in L-threonine deaminase; group B, deficient in the condensing enzyme; group C, deficient in reductoisomerase; group D, deficient in α-β-dihydroxy acid dehydrase. No mutants blocked in the terminal (transaminase) reactions were isolated. Transduction analyses (best-fit, ratio, and complementation tests) with the use of phage 83 established that the linear arrangement of the structural genes is identical with the order of participation of their enzymes in isoleucine and valine biosynthesis, and that these genes comprise a single linkage group which can exist on a single donor fragment during transduction.

The study of isoleucine and valine biosynthesis in *Salmonella typhimurium* (2), *Escherichia coli* (10), *Pseudomonas aeruginosa* (14), *Saccharomyces cerevisiae* (7), and *Neurospora crassa* (17) has led to the recognition of the coordinate nature of the pathways of biosynthesis of these amino acids. In these biosynthetic pathways, each of the last four reactions is controlled by bifunctional enzymes (Fig. 1). Furthermore, in *S. typhimurium* at least the last four of the structural genes which code for the enzymes of isoleucine and valine biosynthesis occur as a single linkage group with a known gene order and orientation within the chromosome map of this bacterium (3, 15).

This report is concerned with the isolation and biochemical characterization of isoleucine- and isoleucine-valine-dependent mutants of *Staphylococcus aureus*, and with the transduction analysis of the linkage relationships of the structural genes which control the biosynthesis of these amino acids.

MATERIALS AND METHODS

Media. In addition to commercially available dehydrated media, a defined synthetic medium was used in this study. The defined broth medium (S broth) was identical to the S broth of Kloos and Pattee (8) except that L-isoleucine, L-valine, and L-leucine were omitted, and L-histidine (20 μg/ml) was added. From S broth, several other defined media were prepared, as follows: S agar consisted of S broth containing 1.5% (w/v) Noble agar (Difco); ES agar consisted of S agar containing 0.05% (v/v) P and D broth (12); preadaptation broth consisted of S broth containing 30 μg of L-isoleucine, 80 μg of L-valine, and 90 μg of L-leucine per ml; derepression broth consisted of S broth containing 2 μg each of L-isoleucine and L-valine per ml. All glassware used with the defined media was cleaned in a mixture of chromic and sulfuric acids and was rinsed thoroughly with deionized water before use.

Bacterial strains and bacteriophages. Strain 655 of *S. aureus* (13), which grows in the absence of exogenous L-isoleucine, L-valine, and L-leucine, served as the parent strain from which all auxotrophic mutants of *S. aureus* were derived. The following strains of *S. typhimurium* were kindly provided by Frank B. Armstrong: strain LT-2; the isoleucine-dependent mutant *ileA12* of strain LT-2, deficient in deaminase activity; and the isoleucine-valine-dependent mutants *ilvA8*, *ilvB10*, and *ilvC16* of strain LT-2, deficient in reductoisomerase, dehydrase, and transaminase activities, respectively, of the isoleucine-valine pathways. These mutants of strain LT-2 have been described in detail (3). All cultures were maintained on Brain Heart Infusion (BHI) Agar (Difco) slants in screw-capped tubes at 4 C. The inocula for all experiments were obtained from subcultures of these stocks, prepared on BHI Agar slants incubated at 37 C for 12 hr and then stored at 4 C. These working stocks were replaced at weekly intervals.

All transduction experiments were performed with phage 83, whereas a total of 19 bacteriophages (29,

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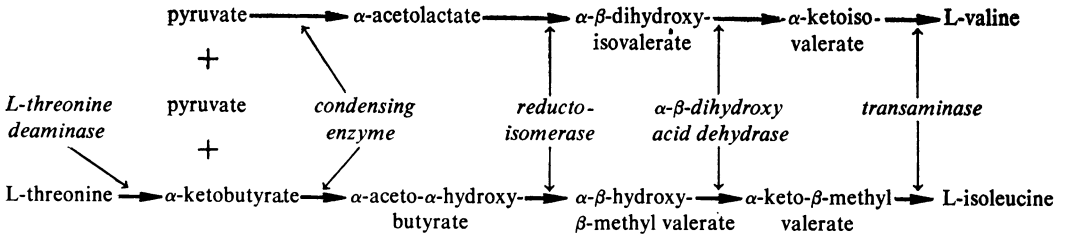

FIG. 1. *Pathway of isoleucine and valine biosynthesis.*

52, 52A, 79, 80, 81, 6, 7, 83, 42B, 47C, 47, 53, 54, 70, 73, 75, 77, and 44A) were employed for phage type determinations. These phages, and the techniques used for their maintenance, propagation, and titration, have been described (12).

Isolation of mutants. The isoleucine- and isoleucine-valine-dependent mutants (*ilv* mutants) of *S. aureus* were obtained after exposure of strain 655 to diethyl sulfate (Bronson and Pattee, *in preparation*). The colonies recovered on Trypticase Soy Agar (TSA, BBL) after mutagenesis were replicated (11) to S agar and TSA plates. After incubation of the replica plates at 37 C for 48 hr, isolates which failed to grow on S agar but which grew well on TSA were inoculated onto BHI Agar slants and incubated at 37 C for 12 hr. The surfaces of duplicate S agar plates were then spread evenly with about 10^7 cells of each isolate contained in 0.1 ml of saline (0.9% NaCl). Several crystals of L-isoleucine and L-valine were then placed together on the surface of one plate, and the second plate received the same additions of amino acids spaced separately. The plates were then incubated at 37 C for 24 hr, and examined for growth of the isolates in response to exogenous L-isoleucine and L-valine.

All isolates were submitted to bacteriophage typing to ascertain their origin as descendants of strain 655 (8).

Auxanography. All auxotrophic mutants used in this study were tested for their growth responses to exogenously supplied intermediates and end products of the biosynthetic pathways of isoleucine and valine by the same methods used to determine their requirements for exogenous isoleucine and valine. Intermediates and end products used were α-keto-isovalerate, α-ketobutyrate, L-valine, L-threonine, α-acetolactate, L-isoleucine, and pyruvate.

Chromatography. All mutants of *S. aureus* were analyzed chromatographically for the accumulation of intermediates of isoleucine and valine biosynthesis in the growth medium. The ability of mutants to accumulate L-threonine was determined in the following manner. The cells from a 12-hr BHI Agar slant culture were suspended in 5 ml of saline, and 0.1 ml of each suspension was then added to separate tubes containing 5 ml of preadaptation broth. These tubes were then shaken in a slanted position at 37 C in a horizontal shaking water bath (WCLID model 2156, Morris Plains, N.J.). After 24 hr, a 0.2-ml sample of each suspension was transferred to a tube containing 5 ml of derepression broth. The derepression broth cultures were shaken for 72 hr at 37 C, and then were stored at 4 C for 48 hr. The cells were then removed by centrifugation (3,400 × *g*, 10 min), and the supernatant fluids were lyophilized to dryness. To remove inorganic salts, each dried residue was resuspended in 0.3 ml of a solution of ethyl alcohol containing 0.5% (v/v) HCl and allowed to stand for 30 min with intermittent shaking. The suspensions were then transferred to separate tubes (13 by 125 mm), which were centrifuged (3,400 × *g*, 10 min) to sediment the precipitated salts. Approximately 0.03 ml of each desalted preparation was applied as a spot to silica gel (Silica Gel H, Brinkmann Instruments, Inc., Westbury, N.Y.) thin-layer chromatography plates. A solution of L-threonine (2 mg/ml) was used as a standard. Each thin-layer plate was developed by the ascending method (5) for 0.5 hr in a solvent composed of acetone-urea-water (60:0.5:40, v/w/v). L-Threonine was detected by spraying the dried chromatogram with Nessler's reagent and then with sodium periodiate (5).

The ability of mutants to accumulate α-keto acids was determined by growing them in preadaptation and derepression broth as described above for the detection of threonine accumulations. The supernatant fluids from the derepression broth cultures were not lyophilized or desalted, because preliminary results showed that both of these procedures precluded the subsequent detection of α-keto acids. Each supernatant fluid (0.5 ml) was spotted on Eaton and Dikeman paper no. 613 (16). A solution of pyruvic acid (100 mg/ml) was used as a standard. The chromatogram was developed (ascending method) for 6.5 hr in a solvent that consisted of *s*-butanol-propionic acid (95:5, v/v) saturated with water (16). The α-keto acids were detected by spraying the chromatogram with *o*-phenylenediamine followed by heating at 100 C for 2 min (5).

Syntrophism. The *ilv* mutants of *S. aureus* were tested for their ability to be stimulated by *ile* and *ilv* mutants of *S. typhimurium*. The cells from 12-hr BHI Agar slant cultures of each mutant of *S. aureus* were suspended in 5 ml of saline, and 0.1-ml samples were spread onto the surfaces of S agar plates. A 0.05-ml sample of the supernatant fluid from derepression cultures of each mutant of *S. typhimurium*, prepared by the same methods used to derepress mutant of *S. aureus*, was then placed on the center of each of the S agar plates. The S agar plates were then incubated at 37 C for 5 days, and were examined daily for evidence of syntrophism (i.e., a zone of growth of the mutant of *S. aureus* surrounding the area inoculated with the culture fluids from the mutants of *S. typhimurium*).

Transduction. The transduction experiments were

performed according to the method of Kloos and Pattee (9), except that the transduction suspensions were prepared at a multiplicity of infection of 1.5, and the cells, after the shaking period, were washed once with 1 ml of saline. They were then resuspended in 1.0 ml of saline, and a 1:10 dilution of these cells was prepared in saline. Portions (0.1 ml) of the diluted cell suspensions were then spread onto duplicate ES agar plates, which were incubated at 37 C. After 48 hr, prototrophic colonies (wild-type transductants) were scored; after an additional 24 hr, the presence or absence of minute colonies (abortive transductants) was determined with the aid of a dissecting microscope. The ratio test (9) was performed by spreading 0.1-ml portions of a 1:30 dilution of the cell suspension onto duplicate ES agar plates supplemented with 10 μg of α-ketobutyrate per ml (added aseptically to ES agar cooled to 42 C). Donor-type and wild-type transductants were scored on these plates after incubation at 37 C for 48 hr.

Designation of mutants. The mutants of *S. aureus* were placed into groups on the basis of biochemical and genetic tests (see Results). With reference to Fig. 1, *ilvA* mutants lacked threonine deaminase activity, *ilvB* mutants were deficient in condensing enzyme activity, *ilvC* mutants were deficient in reductoisomerase activity, and *ilvD* mutants lacked dehydrase activity. The mutants have been designated according to the proposals of Demerec et al. (6).

RESULTS

Isolation of mutants. By means of the replica plating technique, 235 presumed auxotrophic mutants which failed to grow well on S agar were isolated. After more thorough testing, 28 *ilv* mutants were identified and retained for further study.

Biochemical characterization of mutants. By means of auxanography, the *ilv* mutants of *S. aureus* were differentiated into three groups. Group 1 consisted of three mutants (*ilvA4*, *ilvA12*, and *ilvA15*) that responded well to exogenous α-ketobutyrate and L-isoleucine, but not to L-valine or L-threonine; these mutants were apparently deficient in threonine deaminase activity. Four mutants (*ilvB6*, *ilvB17*, *ilvB21*, and *ilvB28*) responded to α-acetolactate and L-valine (in the presence of L-isoleucine), but not to pyruvate. It was concluded that these mutants, placed in group 2, were deficient in the condensing enzyme. Twenty-one mutants responded to α-ketoisovalerate and L-valine (in the presence of L-isoleucine), but not to α-acetolactate. These mutants were placed in group 3.

The supernatant fluids from derepressed cultures of all mutants of *S. aureus* in group 1, and mutant *ileA12* of *S. typhimurium*, contained L-threonine, whereas the remaining mutants of *S. aureus* and *S. typhimurium* failed to accumulate this amino acid. Among the mutants of *S. aureus*, only those in group 2 accumulated detectable amounts of pyruvic acid in the culture medium. Accumulations of threonine and pyruvic acid

TABLE 1. *Summary of biochemical tests used to identify the enzyme deficiencies of ilv mutants of Staphylococcus aureus*[a]

Mutant of *S. aureus*	Test used and results										Enzyme deficiency in mutant of *S. aureus*
	Derepressed culture accumulation	Growth responses to exogenously supplied[b]					Growth responses during syntrophism with *Salmonella* mutants which accumulate				
		ile	ile + val	AKB	AL + ile	KV + ile	thr (*ileA12*)	AL + AHB (*ivbA8*)	DHV + DHI (*ivbB10*)	KV + KI (*ivbC16*)	
ilvA: 4, 12, 15	Thr	+	+	+	+	+	−	+	+	+	Deaminase
ilvB: 6, 17, 21, 28	PYR	−	+	−	+	+	−	+	+	+	Condensing enzyme
ilvC: 2, 3, 5, 8, 10, 11, 14, 16, 19, 22, 23, 24, 26, 27	None	−	+	−	−	+	−	−	+	+	Reducto-isomerase
ilvD: 1, 13, 25, 29, 30 (9, 18)[c]	None	−	+	−	−	+	−	−	−	+	Dehydrase

[a] Abbreviations: thr = L-threonine; ile = L-isoleucine; val = L-valine; PYR = pyruvate; AL = α-acetolactate; AHB = α-aceto-α-hydroxybutyrate; DHV = α-β-dihydroxyisovalerate; DHI = α-β-dihydroxy-β-methyl valerate; KV = α-ketoisovalerate; KI = α-keto-β-methylvalerate; AKB = α-ketobutyrate.

[b] Other materials tested separately which were not utilized by any mutant included val, thr, PYR, and KV.

[c] Mutants *ilvD9* and *ilvD18* responded anomalously during syntrophism. See text.

were obtained at estimated levels of 1 to 10 μg/ml. This range is only approximate, and is based upon the intensity of color, or fluorescence, and the area occupied by the spots on the chromatograms relative to the standards. Culture fluids from *S. aureus* strain 655 and *S. typhimurium* strain LT-2 failed to exhibit either of the above accumulations.

The results of the syntrophism tests, using mutants of *S. typhimurium* to stimulate the growth of *ilv* mutants of *S. aureus*, permitted all mutants of *S. aureus* to be differentiated into four groups. Mutants of *S. aureus*, previously placed into groups 1 and 2 on the basis of auxanography and chromatographic analysis, were stimulated by all mutants of *S. typhimurium* except *ileA12* (which lacks threonine deaminase). Mutants of *S. aureus* in group 3 (as defined by auxanography) were differentiated into two subgroups according to their patterns of stimulation during syntrophism. Mutants of group 3a (*ilvC: 2, 3, 5, 8, 10, 11, 14, 16, 19, 22, 23, 24, 26,* and *27*) were stimulated by mutants *ilvB10* and *ilvC16* of *S. typhimurium* (which lack active dehydrase and transaminase, respectively), whereas mutants of group 3b (*ilvD: 1, 13, 25, 29,* and *30*) were stimulated only by mutant *ilvC16*. On the basis of these results, it was concluded that mutants of group 3a lacked reductoisomerase activity, whereas mutants of group 3b were deficient in dehydrase activity. The only discrepancy between these groupings, defined by biochemical tests, and the final genetic groupings, based on complementation tests and the best-fit analysis, involved mutants *ilvD9* and *ilvD18*, both of which were stimulated by mutants *ilvB10* and *ilvC16* of *S. typhimurium*. In no instance was a mutant of *S. aureus* stimulated by culture fluids prepared from *S. typhimurium* strain LT-2.

The results of the biochemical tests used to characterize the *ilv* mutants of *S. aureus* are summarized in Table 1.

Genetic studies. The methods employed for the genetic analysis were based on those used previously (9). One of the most critical modifications was the choice of the enrichment used to prepare ES agar. Relatively low frequencies of transduction were obtained when S agar was used as the selective medium. The level of enrichment used for the selection of histidine-independent transductants, 1% P and D broth (9), failed completely to permit the isolation of wild-type transductants in the present study, owing to the excessive growth of the background inoculum. Therefore, the effect of the concentration and kind of enrichment used to prepare ES agar was examined in more detail. The results of these experiments (Table 2) reveal the critical nature of the enrichment used to prepare ES agar. The failure of the higher concentrations of BHI, yeast extract, and TSB to support high observed transduction frequencies is attributed to the heavy background growth attained under these conditions.

TABLE 2. *Effect of the nature and concentration of enrichment added to S agar on the frequency of transduction of ilv mutants of Staphylococcus aureus by phage 83/655*[a]

Enrichment[b] and concn (v/v) added to S agar	Frequency of transduction[c] of recipient			
	ilvA4	*ilvB6*	*ilvC3*	*ilvD9*
None	211	137	143	281
P & D				
1.00%	1	0	0	3
0.25%	401	295	275	430
0.05%	1,046	845	853	1,336
0.01%	340	271	269	360
YE				
1.00%	0	0	0	0
0.25%	26	10	8	29
0.05%	540	356	351	635
0.01%	118	98	98	128
TSB				
1.00%	0	0	0	0
0.25%	0	0	0	0
0.05%	3	1	1	2
0.01%	15	8	8	18
BHI				
1.00%	1	0	0	2
0.25%	21	7	5	21
0.05%	110	76	69	115
0.01%	81	55	59	85

[a] Phage 83 prepared on strain 655.

[b] P & D = P and D broth (12). YE = yeast extract (Difco), 0.3% (w/v). TSB = Trypticase Soy Broth (BBL). BHI = Brain Heart Infusion (Difco).

[c] Average number of wild-type colonies recovered from a 0.1-ml sample of a 1:10 dilution of the transduction suspension.

All *ilv* mutants of *S. aureus* were infected with phage 83 previously propagated on strain 655 (phage 83/655); the recipient capacity (incidence of wild-type transductants), reversion frequency, and the incidence of abortive transductants were determined. All mutants, after infection with phage 83/655, gave rise to both wild-type and abortive transductants. That the minute colonies observed were abortive transductants is indicated by: (i) their small size (average diameter, 0.05 mm); (ii) their failure to yield more than a single colony when subcultured on ES agar; and (iii) their absence from transduction suspensions prepared with an *ilv* mutant infected with phage 83 propagated on that mutant, although their presence was consistently observed in all transduction suspensions prepared with phage 83/655

and *ilv* mutants. In addition, preliminary experiments have revealed an apparent "conversion" of abortive transductants to wild-type transductants when phage 83/655 is exposed to low levels of ultraviolet irradiation; these results are very similar to those obtained by Benzinger and Hartman (4) with phage P22.

From among the available mutants, representatives were selected from each biochemical group and submitted to genetic analysis. The mutants which were used were selected on the basis of their low reversion frequencies and minimal leakiness, and on the basis of their strong reactions in biochemical tests.

The order of sites occupied by these mutations and hence the order of the structural genes controlling isoleucine and valine biosynthesis, was determined by the best-fit test (i.e., the order of mutant sites which best fits all frequencies of recombination in reciprocal transductions between all mutants). The results of this experiment (Table 3) reveal a sequence of genes as follows: *ilvA—ilvB—ilvC—ilvD*. The mutants used in the best-fit analysis are listed in their appropriate sequence in Table 3. Two mutants, *ilvC10* and *ilvD29*, exhibited low donor and recipient capacities, whereas the remaining mutants were quite uniformly active in this respect. In this same experiment, the ability of each mutant to complement each other mutant was determined. In all instances, mutants which were members of the same biochemical group failed to complement one another, whereas all mutants complemented all other mutants belonging to a different biochemical group. Thus, the complementation data correlate precisely with the biochemical groupings of these mutants. The genetic data also permitted mutants *ilvD9* and *ilvD18* to be assigned to the proper group.

To verify the positions of the genes more precisely, a ratio test was performed among representative mutants occupying sites within each gene. The experiment was conducted in such a fashion that all *ilvA* mutants (capable of utilizing exogenous α-ketobutyrate) were used as donors, and two mutants of each of the remaining genes (which were unable to utilize α-ketobutyrate) were used as recipients. The results (Table 4) are in agreement with the arrangement of genes as determined from the best-fit test. Owing to the relatively low concentration of α-ketobutyrate used in ES agar during these experiments, the donor-type transductants were clearly differentiated from wild-type transductants on the basis of colony morphology. Representative donor-type transductants, chosen randomly on the basis of their smaller size from each transductant population, were examined genetically and biochemically to verify their genetic identity to the donor from which they were presumably derived. In every instance, the donor-type isolates (i) failed to form recombinants after infection with phage 83 prepared on the appropriate donor, (ii) formed the expected numbers of wild-type recombinants after infection with phage 83/655, and (iii) were capable of utilizing exogenous α-

TABLE 3. *Transduction analysis of the linkage relationship among ilv mutants of Staphylococcus aureus*

Recipient	Reversion frequency	Transduction frequency[a] obtained with indicated donor															
		655	*ilv-A12*	*ilv-A15*	*ilv-A4*	*ilv-B6*	*ilv-B17*	*ilv-B28*	*ilv-C10*	*ilv-C14*	*ilv-C3*	*ilv-C2*	*ilv-D25*	*ilv-D13*	*ilv-D9*	*ilv-D18*	*ilv-D29*
ilvA12	33	1,007	0	1	0	530	551	552	95	618	620	627	847	872	991	1,000	55
ilvA15	3	1,005	0	0	1	500	531	551	88	582	583	592	831	868	987	996	51
ilvA4	0	1,046	1	2	0	499	512	547	80	580	581	586	809	812	975	978	50
ilvB6	3	845	591	580	575	3	0	2	71	370	372	415	546	609	791	795	30
ilvB17	2	909	601	600	595	1	0	0	55	360	368	388	509	595	781	784	17
ilvB28	3	996	608	602	598	0	1	0	36	309	366	380	487	585	751	780	16
ilvC10	1	208	88	86	79	75	44	36	0	2	4	3	111	117	151	162	15
ilvC14	2	827	736	648	590	281	240	237	1	0	3	1	151	181	495	496	15
ilvC3	0	853	765	652	599	285	245	238	2	3	0	0	150	162	457	493	13
ilvC2	2	861	769	697	694	407	386	372	2	1	0	0	114	134	454	473	11
ilvD25	35	998	790	783	781	508	472	455	93	151	146	71	0	4	5	3	5
ilvD13	5	1,129	806	792	785	603	591	584	113	78	68	56	1	0	4	4	1
ilvD9	1	1,336	1,261	1,008	1,004	813	811	707	114	574	544	540	5	4	0	3	2
ilvD18	0	1,777	1,300	1,009	1,006	973	912	807	117	716	707	695	3	2	1	0	1
ilvD29	1	89	59	55	49	28	22	19	19	17	17	15	1	1	0	0	0

[a] Average number of wild-type colonies recovered from a 0.1-ml sample of a 1:10 dilution of the transduction suspension. The transduction frequencies have been corrected for the numbers of wild-type colonies recovered from control suspensions (reversion frequencies).

TABLE 4. *Probability (p) of independent integration of different ilvA alleles into ilv mutants of Staphylococcus aureus*

Recipient	Frequency of independent integration with indicated donor					
	ilvA12		*ilvA15*		*ilvA4*	
	p[a]	No.[b]	*p*	No.	*p*	No.
ilvB6	.591	504	.586	532	.585	536
ilvB17	.608	138	.597	154	.594	158
ilvC3	.823	704	.814	724	.809	736
ilvC2	.829	646	.821	668	.819	676
ilvD9	.835	1,142	.831	1,154	.827	1,170
ilvD18	.858	818	.853	830	.847	842

[a] *p* (probability of independent integration of donor allele) = number of wild-type recombinants divided by the number of wild-type recombinants + donor-type recombinants.

[b] No. = total number of recombinants examined for each determination.

ketobutyrate and required only L-isoleucine to grow well on S agar.

DISCUSSION

On the basis of the results of the biochemical tests, it is concluded that the pathways used by *S. aureus* and *S. typhimurium* for the biosynthesis of isoleucine and valine are virtually identical. The ability of α-ketoisovalerate, in the presence of isoleucine, to support the growth of *ilv* mutants of *S. aureus* suggests the existence of a transaminase reaction as the terminal step, despite the failure to isolate mutants which are blocked in this reaction. The failure to isolate mutants deficient in the transaminase reaction of the pathways may be attributed to the possible existence, in strain 655 of *S. aureus*, of more than one enzyme which is active at this step. Adelberg (1) isolated a transaminase from wild-type *Escherichia coli* and certain *ilv* mutants which served to transaminate α-ketoisovalerate but not α-keto-β-methyl valerate. This transaminase permitted a limited amount of valine synthesis to occur. A similar situation exists in *S. typhimurium*: mutants defective in the transaminase reaction are rarely isolated because of their ability to grow with only isoleucine as the growth supplement (3). Although the data are limited, it is possible that a similar situation exists in *S. aureus*.

The value of intergeneric syntrophism as a method of biochemical analysis cannot be overemphasized. At the conclusion of this study, pairs of *ilv* mutants of *S. aureus* were tested under various conditions for evidence of syntrophism. The results of these tests were not reliable; frequently, pairs of mutants which should have exhibited some evidence of syntrophism failed to do so. In contrast, the results obtained when mutants of *S. typhimurium* were used as the source of the stimulating intermediates were, with the aforementioned exceptions involving two mutants of *S. aureus*, in complete agreement with the biochemical and genetic data.

The sequence of genes within the *ilv* linkage group of *S. aureus* (*ilvA—ilvB—ilvC—ilvD*), based on the results of the best-fit and ratio tests, is different from the gene order in *S. typhimurium*. In *S. typhimurium*, the gene order (using our gene designations for comparisons) is *ilvC—ilvA—ilvD—ilvE* (3, 15), the *ilvE* locus representing the structural gene for transaminase. The absence of auxotrophs affecting the *ilvE* gene precludes determining the location of this gene relative to the remaining structural genes of *S. aureus*. However, the structural gene for the condensing enzyme (*ilvB*) has been located within the *ilv* region of *S. aureus*; the location of the corresponding gene in *S. typhimurium* is thus far unknown.

Despite the failure to detect complementation among the *ilvD* mutants of *S. aureus*, the specific nature of this gene and the mutations which affect it remain in some doubt. Mutants *ilvD9* and *ilvD18*, selected for use in the ratio test, exhibited anomalous behavior during syntrophism. Furthermore, the recombination frequencies obtained when the *ilvD* mutants were crossed with the *ilvC* mutants (Table 3) indicate that mutants *ilvD9* and *ilvD18* possess mutations which are quite close to one another, but which are located at some distance from the mutations possessed by mutants *ilvD13* and *ilvD25*. The characterization of the enzymes required for isoleucine and valine biosynthesis in cell-free extracts of *S. aureus* is now in progress, and the results should clarify this situation.

The isolation of donor-type recombinants in crosses involving *ilvA* mutants and representative mutants from the remaining structural genes clearly demonstrates the existence of all structural genes in a single linkage group which can exist on a single donor fragment during transduction. Unlike the genes of the histidine region of *S. aureus* (9), there is no indication that the transducing fragments which include the *ilv* genes are uniform in their lengths or points of termination, or that the *ilv* region exists near one end of the donor fragment.

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