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Correlation Between Pigment Production and Amino Acid Requirements in *Bacillus subtilis*

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Several *Bacillus subtilis* W-23 auxotrophs were unable to produce wild-type pigment normally on minimal agars supplemented sufficiently for growth. This offers a reliable means for scoring genotypes.

This communication presents a convenient tool for distinguishing the prototrophic wild type from several auxotrophic strains of *Bacillus subtilis* W-23 by differences in colonial pigmentation. Wegman and Crawford (5) described a similar phenomenon for tryptophan auxotrophs of *Chromobacterium violaceum*. Some nonpigmented *B. subtilis* strains were recently characterized as genetically stable, but no auxotrophic correlations were mentioned (R. L. Uffen, Ph.D. Thesis, 1969).

Prototrophic colonies of *B. subtilis* W-23 on minimal 1 agar (3) became red during the second day of incubation at 37 C; later, they stained the neighboring agar. Several W-23 multiply auxotrophic strains used to study the cotransfer of genetic markers (4) were unable to produce pigment normally unless given their amino acid requirements at concentrations above those supporting normal growth. When such strains were transduced so that prototrophs and single auxotrophs appeared together on selective plates, there were mixtures of red and white colonies in the same ratios as prototrophs to single auxotrophs. Replica-plating confirmed this apparent correlation, but further study was required to ascertain whether each supplement level that prevented pigment formation by auxotrophs insured recovery of all prototrophic and auxotrophic transductants or transformants. The six double auxotrophs chosen for special study require pairs of seven different amino acids (Table 1, column 1). These strains are those listed by Tyeryar et al. (4), or are other W-23 double auxotrophs similarly developed and characterized. Singly auxotrophic strains, necessary for reconstructed mixtures, were those from which the corresponding double auxotroph had been developed or were derived from that double auxotroph by transduction with phage SP-15 or SP-10 (1). Transduction mixtures (1, 2) of strain *tyr-2 ile-1* with phage SP-15 provided adequate information for choosing the optimal levels of supplementation indicated in Table 1, but a reconstruction experiment was designed for quantitative work with the other five strains. To insure normal conditions of competition for nutrients with the background population and to produce sufficient numbers of isolated, growing colonies for phenotypic scoring, the reconstruction protocol provided that suitable numbers of each combination of genotypes be plated in mixture with the usual concentration of doubly

TABLE 1. *Level of nutritional supplement that permits phenotypic identification of genotype and full recovery of all prototrophs and single auxotrophs of Bacillus subtilis W-23*[a]

Genotype of doubly auxotrophic strain		Readily distinguishable from prototrophs (A^+B^+) by lack of pigment after 48 to 72 hr of incubation with optimal level of required supplement		Amt (μg) of supplement/ml of agar		
				Test limits	Optimal level	
A^-	B^-	A^-B^+	A^+B^-		A	B
tyr-2	*ile-1*	Yes	Yes	12.5–200[b]	12.5	50
met-3	*ile-1*	No	Yes	12.5–50	50[c]	50
lys-1	*ile-2*	Yes	Yes	12.5–50	25	50
lys-1	*tyr-1*	Yes	Yes	12.5–50	25	12.5
arg-1	*leu-1*	Yes	Yes	12.5–100	50	50
leu-2	*phe-2*	Yes	No	12.5–100	50	50[c]

[a] A^+ = prototrophic, and A^- = auxotrophic, for nutritional supplement A. Prototrophic colonies, wild-type W-23 in reconstruction experiments and double transductants when phage was used, were usually present on each plate as phenotype controls.

[b] Supplements were varied by twofold increments from 12.5 μg to the upper limit tested.

[c] The 50 μg/ml requirement for full numerical recovery masked a slight difference in pigment formation. See text.

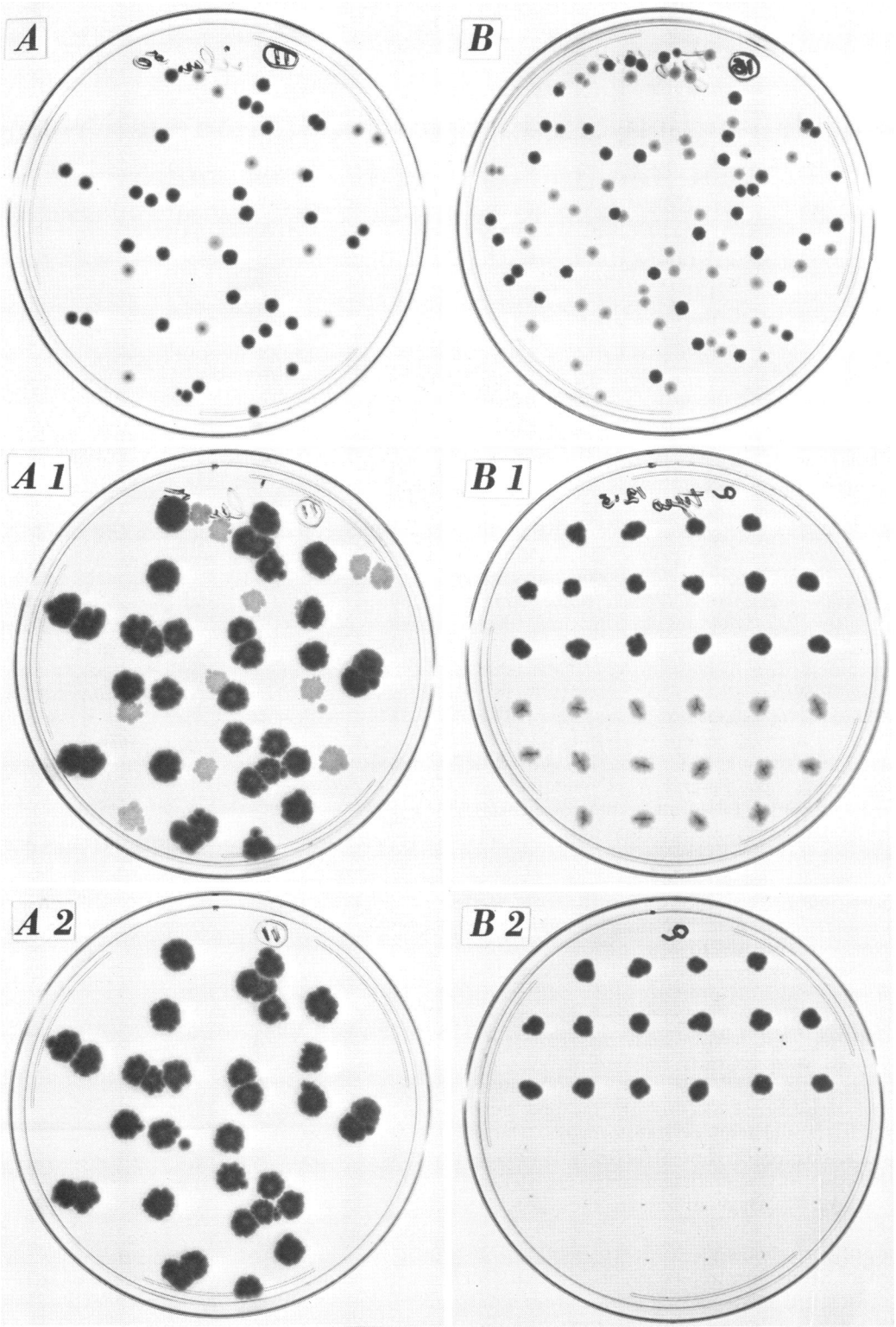

FIG. 1. *Example of phenotypic identification of genotypes occurring in a transduced population of the doubly auxotrophic Bacillus subtilis W-23 tyr-2 ile-1. Transduction mixtures of recipient cells with phage SP-15 were plated on minimal 1 agar plus 50 μg/ml (A) and 12.5 μg/ml (B). A was replicated (velvet template) to isoleucine-supplemented (A1) and unsupplemented (A2) minimal 1 agar. From B, 16 red and 16 white colonies were trans-*

auxotrophic cells on varied levels of each supplement. Care was taken to simulate all aspects of transduction experiments except that the phage was omitted. The selected optimal level of each supplement was always tested in a transduction experiment that included confirmation of the phenotype-genotype correlation by replica-plating.

Table 1 presents the results of these experiments. No level of tyrosine or isoleucine tested affected the frequency of any transductant class (every colony was replicated), but tyrosine concentrations of 25 μg/ml and higher masked the distinction between the tyrosine-requiring singly auxotrophic and the wild-phenotype double transductants. Lysine at 25 μg/ml was adequate for full recovery and gave sharper phenotypic differentiation than did 50 μg/ml. The 50 μg/ml level recommended for the other five amino acids was not inhibitory for any genotype. Replication was necessary for reliable genotypic identification on plates supplemented with methionine or phenylalanine (see Table 1, footnote *c*). We concluded that single auxotrophs with the markers *tyr-1*, *tyr-2*, *ile-1*, *ile-2*, *lys-1*, *arg-1*, *leu-1*, and *leu-2* can form colonies with less supplement than they require for pigment production on plates covered with large numbers of doubly auxotrophic cells. Figure 1 illustrates the phenotypic contrast on selective plates between between colonies whose genotypes were confirmed on replicated plates. Final enumeration and phenotyping are facilitated if colonies are located by an indelible dot on the back of each plate after 20 to 24 hr of incubation, because closely neighboring *Bacillus* colonies tend to coalesce.

Table 2 permits comparison of the cotransfer data for the *tyr-2* and *ile-1* markers when genotype identification was by replica-plating (experiment I) and by phenotypical classification (experiment II) after 48 and 96 hr of incubation. The agreement needs no comment. Although wild-type pigment formation is occasionally delayed beyond 48 hr, error resulting from a 48-hr scoring is probably less than that which results from genotyping by replication when transductant colonies crowd each other. The few questionable or unexpected phenotypes are readily identifiable by replica-picking.

Genotype identification on the basis of pigmentation, where applicable, provides a labor-saving alternative to the usual procedure of replicating to selective media. For any given auxotrophic strain, the optimal levels of supplements and time of incubation giving the best phenotypic differentiation should be determined empirically. Mutants such as we have described may also facilitate the study of pigment synthesis in *B. subtilis* W-23. In this connection, it is noteworthy that Uffen (Ph.D. thesis, Univ. of Massachusetts, Amherst, 1968) implicated L-leucine in pigment synthesis by some strains of *B. subtilis*.

TABLE 2. *Comparison of cotransduction per cent based on identification of transductant genotypes by replica-plating (experiment I) and by colonial pigmentation (experiment II)*[a]

Expt	Transductants			Cotransduction	
	Selected marker	No. of colonies	No. of tyr^+ ile^+[b]	Per cent	Avg per cent
I	tyr^+	592	426	72.0	58.4
	ile^+	923	414	44.9	
II (48 hr)	tyr^+	676	495	73.2	59.3
	ile^+	1483	668	45.0	
II (96 hr)	tyr^+	676	498	73.7	59.4
	ile^+	1483	670	45.2	

[a] Phage SP-15 was used to transduce *Bacillus subtilis* W-23 *tyr-2 ile-1*. This double auxotroph was derived independently of the triply auxotrophic recipient, *lys-1 tyr-1 ile-2*, used by Tyeryar et al. (4) to determine cotransduction per cent for tyr^+ ile^+. The formula for the calculation was the same as theirs.

[b] For experiment I, all colonies on each selective plate were replicated to confirm that all of the red ones were double transductants. For experiment II, the red colonies after 48 and 96 hr of incubation at 37 C were classified as double transductants.

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LITERATURE CITED

1. Taylor, M. J., and C. B. Thorne. 1963. Transduction of *Bacillus licheniformis* and *Bacillus subtilis* by each of two phages. J. Bacteriol. 86:452–461.
2. Taylor, M. J., and C. B. Thorne. 1966. Concurrent changes in transducing efficiency and content of transforming deoxyribonucleic acid in *Bacillus subtilis* bacteriophage SP-10. J. Bacteriol. 91:81–88.
3. Thorne, C. B., and H. B. Stull. 1966. Factors affecting transformation of *Bacillus licheniformis*. J. Bacteriol. 91:1012–1020.
4. Tyeryar, F. J., Jr., M. J. Taylor, W. D. Lawton, and I. D. Goldberg. 1969. Cotransduction and cotransformation of genetic markers in *Bacillus subtilis* and *Bacillus licheniformis*. J. Bacteriol. 100:1027–1036.
5. Wegman, J., and I. P. Crawford. 1968. Tryptophan synthetic pathway and its regulation in *Chromobacterium violaceum*. J. Bacteriol. 95:2325–2335.

ferred with a sterile toothpick to tyrosine-supplemented (B1) and unsupplemented (B2) minimal 1 agar. All of the dark (red-pigmented) colonies grew on unsupplemented plates A2 and B2, indicative of prototrophy. The unpigmented colonies on A and B grew on A1 and B1 respectively, but not on A2 and B2, thus demonstrating that they were "single" transductants requiring isoleucine or tyrosine. Plates were photographed after 48 hr at 37 C.