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# Pleiotropic Menaquinone-Deficient Mutant of *Bacillus subtilis*

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A multiple aromatic amino acid auxotroph of *Bacillus subtilis* 168 has been isolated which is unable to synthesize menaquinone-7 (MK-7) unless supplied with shikimic acid (SHK). The mutant, RB163, was isolated by selecting for resistance to low levels (1.5 μg/ml) of kanamycin. Enzymatic and genetic analyses show that the strain is an *aroD* mutant lacking 5-dehydroshikimate reductase. Under growth conditions in which its MK-7 deficiency is expressed, RB163 is deficient in cytochromes *a*, *b*, and *c*, exhibits low growth yields, and does not sporulate. Genetic analysis indicates that this pleiotropic phenotype is the result of a single genetic event. All phenotypic characteristics are reversible when the mutant is grown under conditions such that MK is synthesized. Comparison of strain RB163 with other *aro* mutants blocked before SHK ("early-*aro*" mutants) reveals interesting differences. Most early-*aro* mutants are cytochrome- and MK-sufficient, sporogenous, and sensitive to kanamycin when grown in the absence of SHK. However, in addition to strain RB163, two other *aro* mutants were found to show the pleiotropic phenotype. These three mutants have in common, and differ from other early-*aro* strains in, the inability to synthesize MK. It is suggested that the phenotypically wild-type *aro* mutants are bradytrophic, allowing enough substrate flow through the common aromatic pathway to satisfy the MK requirement. The pleiotropic mutants are thought to be completely blocked in the common pathway, thus accounting for their inability to synthesize MK.

Bacterial sporulation is a differentiation process that offers a unique system for the study of membrane changes during cellular morphogenesis. Morphological studies show that marked changes occur in the gross structure of the cell and particularly in the architectural arrangement of the cytoplasmic membrane during the transition from vegetative cell to mature spore. It is possible that these morphological alterations may be associated with functional modifications of the membrane. The study of such functional modifications and their importance to sporulation can be approached in two ways. (i) One might measure certain membrane-associated activities (e.g., redox reactions or active transport) during growth and sporulation. Significant changes in these activities during the transition may indicate extensive modifications of membrane structure and composition that are important to the differentiation process. This type of analysis has implicated the tricarboxylic acid cycle enzymes in the early stages of sporulation (10, 11, 13, 14, 34). (ii) Mutants deficient in these membrane-associated activities may be isolated. By analyzing the ability of such mutants to sporulate, the importance of these activities to sporogenesis can be assessed. The failure of many tricarboxylic acid cycle mutants to sporulate at normal frequencies (3, 22) affirms the general value of this approach.

One class of membrane-associated activities is the electron transport system of *Bacillus subtilis*. Included within this system are five cytochromes (4) and menaquinone (MK)-7(7, 24). The work of Chaix and Petit (4) clearly showed that definite changes in the membrane-bound cytochrome composition of *B. subtilis* accompany the transition from vegetative growth to stationary phase. Cytochromes $c_1$ and *o*, predominant during vegetative growth, are replaced by cytochromes *a*, *b*, and *c* during the transition to stationary phase. The apparent

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importance of the latter heme proteins is demonstrated by the inability of certain cytochrome *a*-deficient mutants of *B. subtilis* to sporulate (H. W. Taber and E. Freese, submitted for publication).

Characteristic changes in the concentrations of membrane-associated MK-7 have also been noted during the transition from vegetative growth to early sporulation (S. K. Farrand and H. W. Taber, manuscript in preparation).

Although quinone-deficient mutants have been described for a number of different species (5, 25, 26, 27, 33, 38), no strains of *B. subtilis* showing this phenotype have, until recently, been reported (S. K. Farrand and H. W. Taber, Bacteriol. Proc., p. 161, 1971). Such mutants could prove extremely valuable in determining the functional importance of MK during growth and sporulation. We report here a characterization of the first MK-deficient mutant of *B. subtilis* to be described.

## MATERIALS AND METHODS

**Bacterial strains.** The *B. subtilis* strains used in this investigation are listed in Table 1.

**Chemicals.** All chemicals were of the highest grade available and were not further purified. Solvents for thin-layer chromatography (TLC) and ultraviolet spectrophotometry were spectral grade. Diethyl ether was purchased in and stored in glass bottles. MK-7 was the gift of O. Isler, Hoffman La Roche, Basel, Switzerland. Erythrose-4-phosphate, phosphoenol pyruvate, 3-deoxy-D-*arabino*heptulosonic acid-7-phosphate (DAHP), and dehydroquinate (DHQ) were the generous gifts of E. W. Nester, University of Washington, Seattle.

**Media.** The minimal salts medium (MG) was that of Anagnostopoulos and Spizizen (1) supplemented with 0.5% glucose. MG agar was prepared by adding 1 liter of sterile 4% agar to a sterile 1-liter portion of double-strength MG. Tryptose blood agar base medium (TBAB) was prepared as previously described (34). TBABG medium was prepared by adding sterile glucose to a final concentration of 0.2% to previously autoclaved TBAB medium. T medium (TM), the liquid equivalent of TBAB medium, contained in grams per liter: tryptose (Difco), 10; beef extract (Difco), 3.0; and NaCl, 5.0. The components were dissolved, autoclaved, and stored in glass bottles. Tryptose-glucose medium (TMG) was prepared as above and supplemented with 0.2% glucose. The nutrient sporulation medium was that of Schaeffer (28). AK sporulation medium was prepared by dissolving 30.8 g of dehydrated AK medium #2 (BBL) in 1 liter of distilled water and autoclaving.

**Growth.** To estimate growth, cells were cultured in MG, TM, or TMG media. Samples (10–25 ml) of medium contained in 250-ml nephelometer flasks were inoculated to an initial optical density at 540 nm ($OD_{540}$) of 0.05 to 0.15 from overnight cultures grown in the same medium. Cultures were incubated at 37 C with rapid shaking (200 rpm) to insure optimal aeration. Growth was measured turbidometrically at 540 nm in a Spectronic 20 colorimeter. For batch cultures of 500 to 1,000 ml in volume, cells were grown in 2,800-ml Fernbach flasks with vigorous shaking (150-200 rpm) at 37 C.

**MK-7 assays.** Cells were cultured in 500- or 1,000-ml batches of TMG until 1 h ($T_1$) after the end of exponential growth ($T_0$). Cells were harvested by centrifugation, protoplasts were prepared, and membranes were obtained essentially as described by Salton and Schmitt (24).

Membrane lipids were extracted by either of two procedures. Method A (24) involved repeated acetone-methanol (7:2, vol/vol) extractions. Method B (9) involved a single acetone extraction followed by partition into diethyl ether. Control experiments showed that method B resulted in the recovery of 97% of the MK-7 extractable by method A.

After extraction by either of the above methods, MK-7 was purified by TLC on silica gel G plates (250 $\mu$m thickness) with isooctane-diethyl ether (100:30, vol/vol) as the mobile phase (24). Sulfuric acid charring showed this system to effectively separate MK-7 from other constituents in the lipid extracts. MK-7 was seen on the TLC plates as absorbing spots ($R_f$ = 0.8) when viewed under a hand-held short-wavelength (260 nm) ultraviolet light source. For quantitation, the light-absorbing regions were scraped off the plates and the MK-7 was eluted from the silica gel with three 10-ml portions of diethyl ether. The ether eluates were pooled and filtered to remove residual silica gel, and the solvent was evaporated off at 37 C under a stream of dry nitrogen gas. The isolated MK-7 was redissolved in exactly 3.0 ml of isooctane. The absorption spectra of such preparations were measured between 400 and 200 nm in a Unicam SP 800 recording spectrophotometer (Philips Electronics Instruments, Mount Vernon, N.Y.). Path-length quartz cuvettes (1 cm) with Teflon stoppers were used to prevent evaporation of the solvent. The extinction coefficient at the 248-nm absorption maximum was taken to be $1.9 \times 10^4$ liters $\cdot$ mol$^{-1}$ $\cdot$ cm$^{-1}$ (24). Authentic MK-7 was used to establish chromatographic identity and spectral purity.

**Cytochrome determinations.** For rapid estimation of cytochrome concentrations, 24- or 48-h-old cultures grown in TBAB or TBABG media were observed at liquid nitrogen temperature in a Hartree microspectroscope (29). Wavelength maxima for each of the cytochrome components were: cytochrome *a*, 602 nm; *b*, 561 nm; *o*, 557 nm; $c_1$, 553 nm; and *c*, 549 nm (35). Relative cytochrome concentrations were expressed on a scale of from 0 (absent) to 5 (maximal) on the basis of the heme protein component showing the highest absorption in the spectroscope. Comparison with spectrophotometric measurements (see below) showed that the spectroscope technique was accurate to within about $\pm$10%.

Absolute absorption spectra of the different cytochrome components were determined at liquid nitrogen temperature in a Cary model 14 spectrophotometer (Cary Instruments, Monrovia, Calif.) equipped with scattered transmission and period pen response

TABLE 1. *List of strains*

| Strain | Genotype | Relevant enzyme defect or phenotype | Source |
|---|---|---|---|
| RB1[a] | *trpC2* | INGP synthase | Laboratory strain originally derived from strain 168 |
| RB2 | Prototroph | | RB1 made Trp$^+$ by transformation with W23 DNA |
| WB746 | Prototroph | | E. Nester. A spontaneous revertant of strain 168 |
| RB163 | *aroD* | DHS reductase | Spontaneous kanamycin-resistant derivative of RB1. |
| | *trpC2* | Kanamycin resistant | |
| SB163A[b] | *aroA* | DAHP synthase | E. Nester |
| | *trpC2* | | |
| | *trpC2* | Chorismate mutase 1,2 | |
| SB163B[b] | *aroH* | DAHP synthase | E. Nester |
| | *aroA* | | |
| | *trpC2* | | |
| SB165 | *aroB* | DHQ synthase | E. Nester |
| | *trpC2* | | |
| SB167 | *aroB* | DHQ synthase | E. Nester |
| | *trpC2* | | |
| SB202 | *aroB* | DHQ synthase | E. Nester |
| | *trpC2* | | |
| | *tyrA* | Prephenate dehydrogenase | |
| | *hisB* | Requires histidine | |
| JH465 | *aroB* | DHQ synthase | J. A. Hoch |
| | *trpC2* | Kanamycin resistant | |
| SB121 | *aroC* | DHQ dehydratase | E. Nester |
| | *trpC2* | | |
| SB120 Trp$^+$-1 | *aroD* | DHS reductase | E. Nester |
| WB719 | *aroD* | DHS reductase | E. Nester |
| WB2438 | *aroG*[c] | Chorismate mutase 3 | E. Nester |
| | | Kanamycin resistant | |
| $Mu_6u_5u_{16}$ | *purA* | Requires adenine | N. Sueoka |
| | *leuA* | $\alpha$-IPM synthase | |
| | *metB* | Requires methionine | |
| BD71 | *argC* | Requires arginine | D. Dubnau |
| | *pyrA* | Requires pyrimidines | |
| | *hisA* | Requires histidine | |
| BD40 | *argA* | Requires arginine | D. Dubnau |
| | *pheA* | Prephenate dehydratase | |
| GSY189 | *trpC2* | | C. Anagnostopoulos |
| | *thr* | Requires threonine | |
| GSY240 | *metB* | Requires methionine | C. Anagnostopoulos |
| | *ilvA* | Threonine deaminase | |
| GSY390a | *argA* | Requires arginine | C. Anagnostopoulos |
| | *lys* | Requires lysine | |

[a] All derivatives of strain 168 also lack the *aroH* gene product, chorismate mutase 1,2 (see ref. 17).

[b] Strain SB163 segregated into two strains, one containing the *aroH* gene product and one lacking it. The reason for this is unknown (E. Nester, personal communication).

[c] As a result of the *aroG* mutation, this strain is also defective in DAHP synthase and SHK kinase activities (E. Nester and W. Nakatsukasa, personal communication).

accessories. Dithionite-reduced whole cell pastes were transferred to a Plexiglas cuvette of special design and frozen in liquid nitrogen. The low-temperature cuvette was transferred to an unsilvered Dewar flask containing liquid nitrogen, which had been placed in close proximity to the photomultiplier tube. The light scattering was compensated for by placing a sheet of polyethylene (3 mm thickness) at the entrance of the reference beam. Spectra were measured between 630 and 510 nm at a chart speed of 2 in/min, a scan speed of 0.25 nm/s, and the period pen response at setting 5. Wavelength maxima for the cytochrome components were in good agreement with the microspectroscope.

**Antibiotic resistance.** Strains were rapidly screened for low-level antibiotic resistance by streaking on TBAB medium supplemented with 1.5 $\mu$g of

kanamycin per ml. Plates were incubated at 37 C and examined for growth after 24 and 48 h.

For determining the effect of nutritional supplements on antibiotic resistance, a split parallel culture technique was used. TMG medium (25 ml), with or without supplements, was inoculated to an initial $OD_{540}$ ≈0.05 from overnight cultures grown in the same medium. The cultures were incubated at 37 C with vigorous shaking. When the cultures were in early exponential phase ($OD_{540}$ = 0.15), 10-ml samples were removed and transferred to sterile, prewarmed nephelometric flasks containing enough kanamycin in 0.15 ml of water to give a final drug concentration of 1.5 μg/ml. All cultures were then incubated as above and growth was followed at 15-min intervals for at least 2 h after $T_0$.

**Enzyme assays.** Cells were grown in 600-ml batches of MG medium supplemented as noted in the text at 37 C with rapid shaking. Cultures were harvested at the end of exponential growth, and soluble cell extracts were prepared by sonic oscillation (21). The crude extracts were applied to Sephadex G-25 columns equilibrated with either 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5 (DAHP synthase, DHQ dehydratase assays), or 0.05 M Tris-hydrochloride, pH 8.2 (DHQ synthase, 5-dehydroshikimate [DHS] reductase assays). The extracts were eluted with the corresponding buffer and either assayed immediately or stored at −45 C.

DAHP synthase (EC 4.1.2.15) was assayed by the method of Srinivasan and Sprinson (31) by using an extinction coefficient for the β-formylpyruvate derivative at 549 nm of $7.2 \times 10^4$ liters · $mol^{-1} \cdot cm^{-1}$. Specific activity was expressed as nanomoles of DAHP formed per minute per milligram of protein. DHQ synthase was assayed by the method of Srinivasan et al. (30). Specific activity was expressed as nanomoles of DAHP removed per minute per milligram of protein. DHQ dehydratase (EC 4.1.1.10) was assayed by the procedure of Mitsuhashi and Davis (20) by using an extinction coefficient for the product (DHS) of $1.9 \times 10^4$ liters · $mol^{-1} \cdot cm^{-1}$. Specific activity was expressed as nanomoles of DHS formed per minute per milligram of protein. DHS reductase (EC 1.1.1.25) was assayed in the reverse by a modification (21) of the procedure of Yaniv and Gilvarg (37). The extinction coefficient for reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm was taken to be $6.2 \times 10^3$ liters · $mol^{-1} \cdot cm^{-1}$, and specific activity was expressed as nanomoles of NADPH formed per minute per milligram of protein.

**DNA-mediated transformation.** Biologically active deoxyribonucleic acid (DNA) was prepared essentially as described by Saito and Miura (23). Transformations were performed by the method of Boylan et al. (2). Total viable counts were determined by plating on MG agar supplemented with the nutritional requirements of the recipient strain. Transformants were determined by plating on MG agar supplemented as noted above. All plates were incubated for 24 to 96 h at 37 C, and plates were not discarded unless no growth appeared after 4 days.

**PBS1-mediated transductions.** Second-passage transducing lysates were prepared by a method developed in this laboratory (S. K. Farrand, Ph.D. thesis, Univ. of Rochester, Rochester, N.Y., 1972). Lysates were not titered for lytic particles since those giving satisfactory transduction ($\geq 10^3$ transductants/ml) were considered suitable for use.

Phage PBS1-mediated two-factor transductions were performed following the suggestions of David Dubnau. Briefly, 10 ml of VY medium cultures (25 g of dehydrated veal infusion powder +5 g of yeast extract/liter of water) of the recipient strains were grown at 37 C with shaking until phase contrast microscopy showed most of the culture to be motile ($OD_{540} \simeq 1.2$–$1.8$). For each cross, 2.0 ml of the culture was pipetted into sterile 50-ml glass tubes containing 1.0 to 2.0 ml of the appropriate phage lysate. The mixtures were left standing at room temperature for 10 min to allow phage absorption. The tubes were then shaken gently at 37 C for 30 min, and the cultures were transferred to sterile, small, plastic tubes and centrifuged at 10,000 × *g* for 5 min. Supernatant fluids were discarded and the cell pellets were resuspended in 2.0 ml of MG medium. Samples (0.2 ml) of the appropriate dilutions were plated by spreading on MG agar supplemented as noted above. Plates were incubated and colony counts were performed as described for the transformation experiments.

**Protein determinations.** Protein concentrations were determined by the method of Lowry et al. (18) with crystalline bovine serum albumin as the standard.

## RESULTS

**Growth requirements of strain RB163.** Previously, a large number of spontaneous respiration-deficient mutants of *B. subtilis* strain 168 were isolated by selection for low-level resistance to kanamycin (H. W. Taber, P. Bitoun, and G. Halfenger, Bacteriol. Proc., p. 139, 1970). One such strain, RB163, showed a strict growth requirement for the aromatic end products (Aro), tryptophan, tyrosine, phenylalanine, and *p*-aminobenzoic acid (PABA). Under such conditions, or when grown on TBAB medium, the mutant grew very poorly as compared with the parent strain, RB1. Inclusion of 0.2% glucose in TBAB medium was stimulatory, but growth, as indicated by colony diameter, was reduced compared with the parent strain. The strict Aro requirement, coupled with the observation that SHK could replace tyrosine, phenylalanine, and PABA, and restore growth to the parental level (S. K. Farrand and H. W. Taber, Bacteriol. Proc., p. 161, 1971) suggested that strain RB163 was blocked in one of the first four reactions of the common aromatic amino acid pathway (Fig. 1).

The growth characteristics of strain RB163 were compared with those of the parent strain and an *aroB* mutant, SB202. When grown in MG medium + Aro, strains SB202 and RB1

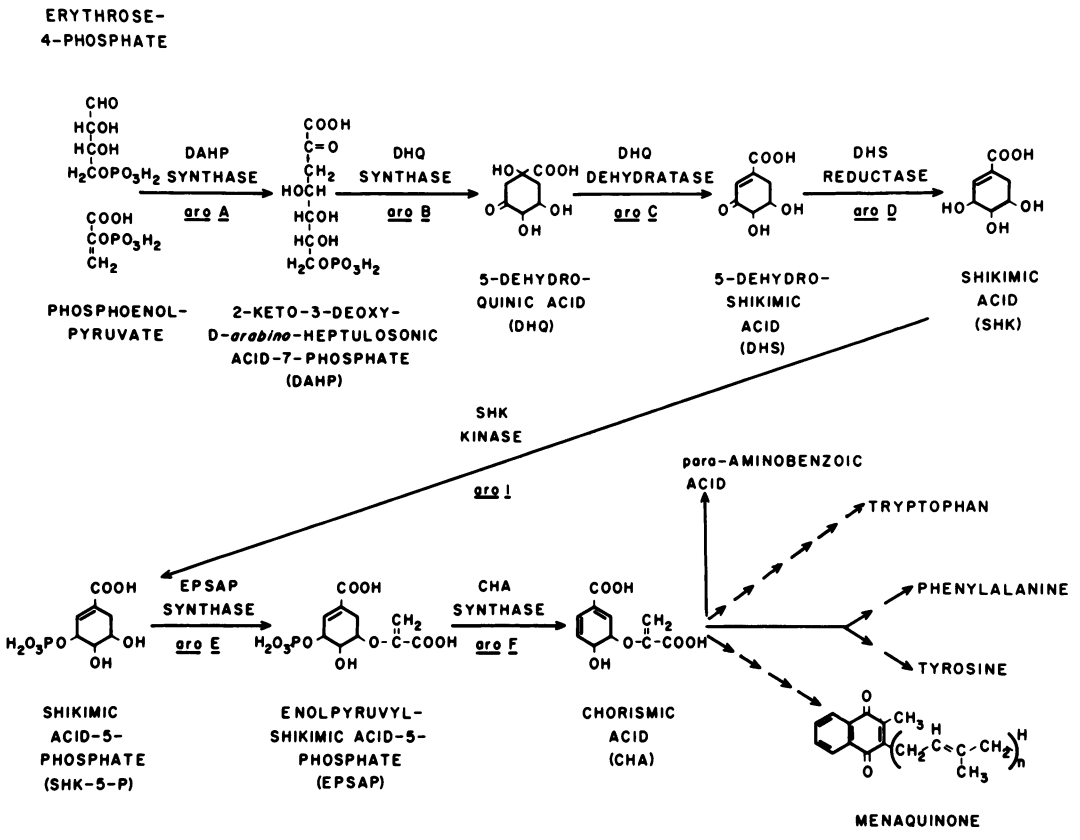


FIG. 1. *Common aromatic amino acid biosynthetic pathway. Common enzyme names appear above the reaction arrows. Appropriate gene designations appear below the arrows (21).*

showed essentially identical growth characteristics (Fig. 2A). Strain RB163, although showing approximately the same vegetative growth rate, differed in that it deviated from exponential growth earlier and reached a substantially lower final yield. Inclusion of SHK in the medium resulted in growth rates and final yields of strain RB163 indistinguishable from strains RB1 and SB202 (Fig. 2B).

**Cytochrome complement of strain RB163.** Low-temperature microspectroscope analysis has shown that many small-colony mutants of *B. subtilis* obtained by selection for resistance to kanamycin exhibit cytochrome abnormalities (35). Microspectroscope and spectrophotometry observations on frozen intact cells of strain RB1 grown on TBABG medium for 24 h revealed the typical cytochrome complement exhibited by wild-type stationary phase cultures (Fig. 3, top). Cytochromes *a* (602 nm), *b* (560 nm), and *c* (549 nm) predominate, and cytochromes $c_1$ (553 nm) and *o* (557 nm) are low. This is clearest in the microspectroscope observations, in which

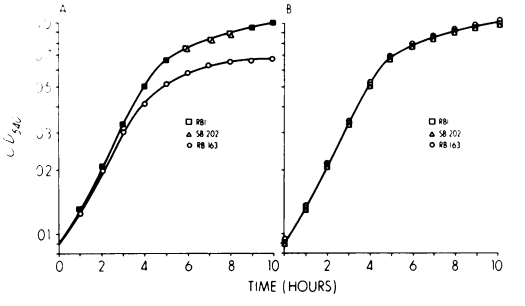


FIG. 2. *Growth characteristics of strains RB1, RB163, and SB202 cultured in MG medium. Panel A, MG + tryptophan, tyrosine, phenylalanine, and PABA. Amino acids were supplied at 25 μg/ml. PABA was supplied at 5 μg/ml. Panel B, MG medium + shikimate (25 μg/ml). Cultures of strain SB202 were also supplemented with histidine (25 μg/ml).*

practically no absorption bands corresponding to cytochromes $c_1$ or *o* can be observed. In the recorded spectra, $c_1$ and *o* do not appear to be lower in concentration than are *c* and *b*. How-

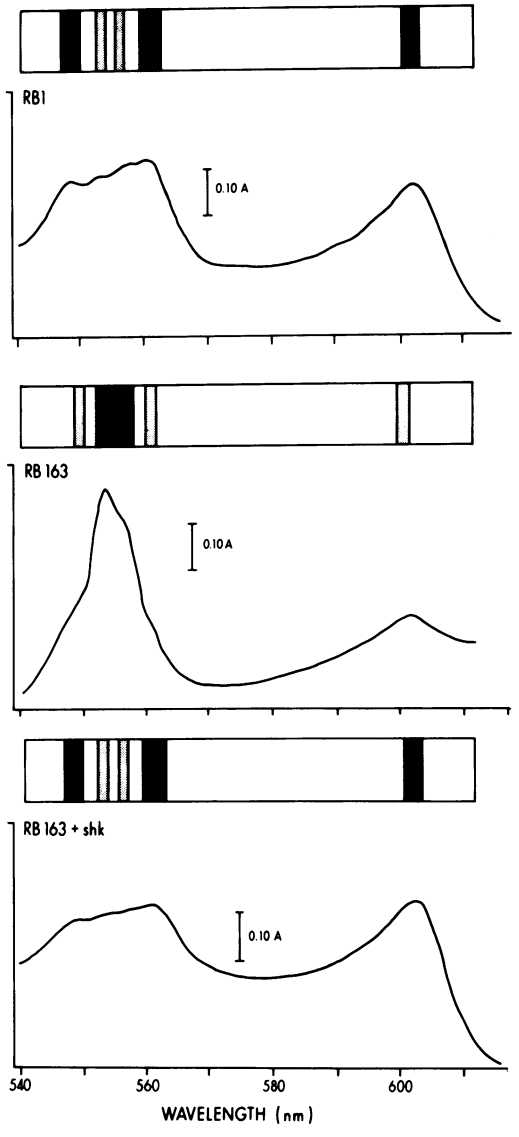


FIG. 3. *Low-temperature visible cytochrome absorption spectra of strains RB1 and RB163. Cells were grown for 24 h on TBABG medium. Bar at top represents the absorption patterns observed in the Hartree microspectroscope. The bottom portion of each panel shows the spectrophotometric recordings. Spectra were determined on dithionite-reduced whole cell preparations as described in Materials and Methods. Shikimate was supplied at 50 μg/ml.*

ever, it is likely that much of the absorbancies at 553 and 557 nm can be attributed to the shoulders of the 549 (*c*) and 560 nm (*b*) peaks.

A strikingly different pattern was observed for strain RB163. Under identical conditions, cytochromes *a*, *b*, and *c* were low, whereas the concentrations of $c_1$ and *o* remained elevated (Fig. 3, center). Such a spectrum is characteristic of exponential cultures (4). However, after prolonged incubation periods the spectrum of strain RB163 was unchanged. Since SHK restored normal growth properties to strain RB163, the effect of this compound on the cytochrome composition was determined. Inclusion of SHK at 50 μg/ml in TBABG medium resulted in complete restoration of the parental type stationary-state cytochrome phenotype (Fig. 3, bottom). SHK had no effect on the cytochrome complement of strain RB1.

**Kanamycin resistance.** Strain RB163 was originally selected for by its resistance to a low level (1.5 μg/ml) of kanamycin. The effect of SHK upon this resistance was determined by plating the mutant on both SHK-supplemented (25 μg/ml) and unsupplemented TBABG medium, both containing 1.5 μg of kanamycin per ml. The results show that, in the absence of SHK, strain RB163 maintained its resistance to the antibiotic, whereas strain RB1 was highly sensitive (Table 2). Inclusion of SHK in the medium had no effect on the response of strain RB1 but rendered the mutant sensitive to the antibiotic.

**Sporulation properties.** Because many perturbations of systems involved in energy metabolism result in oligosporogeny or asporogeny (3, 11, 34, H. W. Taber and E. Freese, submitted for publication), strain RB163 was assayed for ability to sporulate both in the presence and absence of SHK. The mutant could be clearly distinguished from its parent both by its growth

TABLE 2. *Growth and sporulation characteristics and response to kanamycin of strains RB1 and RB163*

| Strain | Additions[a] | Colony size[b] (cm) | Spores[c] (%) | | Response to kanamycin[d] |
|---|---|---|---|---|---|
| | | | Nutrient sporulation medium | AK medium | |
| RB1 | None | 1-2 | >50 | >50 | Sensitive |
| | SHK | 1-2 | >50 | >50 | Sensitive |
| RB163 | None | 0.1-0.2 | <0.1 | <0.1 | Resistant |
| | SHK | 1-2 | >50 | >50 | Sensitive |

[a] SHK was supplied at 25 μg/ml.

[b] Determined after 24 h of growth at 37 C on TBAB, nutrient sporulation, and AK media.

[c] Colonies were picked from plates grown for 72 h and emulsified in a drop of water on microscope slides. Refractile spores were estimated by phase contrast microscopy.

[d] Determined after 24- and 48-h periods of incubation on TBABG or TBABG + SHK, both supplemented with 1.5 μg of kanamycin per ml.

characteristics and by its inability to sporulate on unsupplemented media (Table 2). Strain RB163 grew poorly on both nutrient sporulation and AK media, but when supplemented with SHK, colony diameter was similar to that of strain RB1. Although the values for sporulation are only approximate, no spores were seen in any preparations of strain RB163 grown without SHK. However, inclusion of SHK in the medium allowed the mutant to sporulate with a frequency approximating that of strain RB1 (Table 2).

**Quinone analysis of strain RB163.** Reduced growth in the presence of aromatic supplements, together with the observation that SHK could replace these supplements and restore growth, cytochrome, and sporulation characteristics to parental levels, indicated that the aromatic supplements were not entirely satisfying the nutritional requirements introduced by the mutation in strain RB163. Since MK-7 is biosynthesized by a series of reactions branching from the common aromatic supplement pathway at the level of chorismate (6, 16), the mutant phenotype could have been due to a deficiency in this lipid-soluble electron transport component.

Strain RB163 was assayed for its ability to synthesize MK-7 both in the presence and absence of SHK. The quinone concentrations derived from the ultraviolet spectra are presented in Table 3. It is clear that when strain RB163 is grown in the absence of SHK, it is capable of synthesizing less than 1/1,000 of the amount of MK-7 synthesized by the parent strain. Inclusion of SHK in the growth medium restored MK-7 concentrations to normal levels in the mutant as compared to strain RB1. It should be noted that no MK-7 was detected in lipid extracts of strain RB163 grown in the absence of SHK either by TLC (Fig. 4) or by spectrophotometry.

TABLE 3. *MK concentrations in strains RB1 and RB163*

| Strain | Addition[a] | Culture vol[b] (ml) | Membrane recovered (mg) | Total MK recovered[c] (nmol) | nmol of MK/mg of membrane |
|---|---|---|---|---|---|
| RB1 | None | 500 | 66.3 | 363 | 5.4 |
| RB163 | None | 1,000 | 100.7 | <0.48[d] | <0.005 |
| | SHK | 500 | 64.5 | 358 | 5.5 |

[a] SHK was supplied at 50 μg/ml of TMG medium.
[b] Cultures were all harvested 1 h after the end of the exponential growth.
[c] Lipids were extracted from lyophilized membranes by method A (24).
[d] Below the sensitivity of the assay.

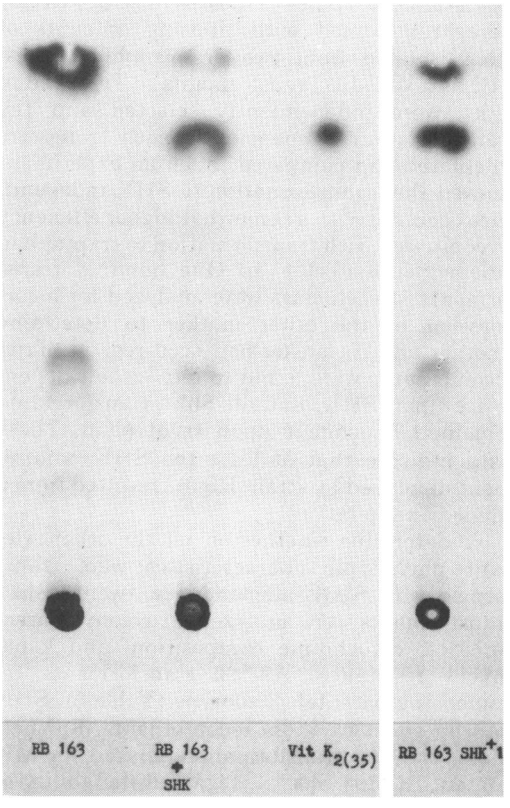


FIG. 4. *Thin-layer chromatogram of lipid extracts from strains RB163 and RB163 Shk+-1. Chromatography was carried out on silica gel G plates with isooctane-diethyl ether (100:30, vol/vol) as the mobile phase. Components were visualized by spraying with sulfuric acid (5% in ethyl alcohol) followed by charring at 100 C for 10 min. From left to right, samples were: total lipid extract from membranes of strain RB163 grown in 1,000 ml of TMG medium; total lipid extract from membranes of RB163 grown in 500 ml of TMG medium + SHK (50 μg/ml); authentic MK-7 (vitamin K2(35)); total lipid extract from strain RB163 Shk+-1 grown in 500 ml of TMG medium. All cultures were harvested at T1 and lipids were extracted by method B.*

**Genetic analysis of the pleiotropic phenotype.** An inability to obtain spontaneous revertants together with the pleiotropic phenotype of strain RB163 suggested that the strain was multiply mutated. However, reversal of the pleiotropic characteristics by growth in the presence of a single nutritional supplement (SHK) argued against this interpretation.

The problem was further investigated by genetic analysis by using DNA-mediated trans-

formation. Competent cultures of strain RB163 were transformed with limiting amounts of DNA isolated from two prototrophic strains, RB2 and WB746. Transformants to Trp$^+$ and to Shk$^+$ were independently selected and the transformation frequencies to each trait were calculated and compared. Such an experiment showed that transformation to SHK independence occurred with a somewhat higher efficiency as compared with transformation to tryptophan independence (Table 4). One hundred transformants of each class were analyzed for incorporation of the other marker to determine whether any cotransfer had occurred. All Trp$^+$ recombinants were found to retain their dependence upon SHK, and all Shk$^+$ transformants remained dependent upon tryptophan. These data indicate that at least the SHK requirement displayed by strain RB163 resulted from a single genetic lesion.

To determine whether or not the other relevant phenotypic characteristics were transformed with SHK-independence, twenty Shk$^+$ transformants were analyzed for colony characteristics, cytochrome composition, and kanamycin resistance. All 20 transformants regained the parental phenotype (Table 5). SHK had no effect on these recombinants. Furthermore, the one transformant analyzed for MK content, RB163 Shk$^+$-1, regained the ability to synthesize MK-7 when grown in the absence of SHK (Fig. 4). In a similar manner, 20 transformants to Trp$^+$ were found to retain the pleiotropic phenotype exhibited by strain RB163 (data not shown).

**Enzymatic analysis of the aromatic block in strain RB163.** It seemed clear from the preceding results that an enzymatic block in the Aro pathway was responsible for the quinone deficiency and, most probably, also for the other phenotypic abnormalities exhibited by strain RB163. To determine the exact location of the block, the first four enzyme activities of the common pathway were assayed (Fig. 1). In addition, strains RB1, RB163 Shk$^+$-1, and appropriate *aro* mutants were assayed as controls. The results (Table 6) indicate that strain RB163 appears to be slightly derepressed for the first three activities, showing levels twofold higher than those in the parent strain. However, within the sensitivity of the assay, the mutant contains no DHS reductase activity, the product of the *aroD* gene (21). In addition, strain RB163 Shk$^+$-1 regained DHS reductase activity as a consequence of transformation to SHK independence.

TABLE 4. *Transformation frequency analysis of the aro mutation in strain RB163*

| Donor DNA | Transformation frequency | | SHK$^+$: Trp$^+$ |
|---|---|---|---|
| | Trp$^+$ [a] | SHK$^+$ [b] | |
| RB2 (proto) | $9.01 \times 10^{-4}$ | $2.2 \times 10^{-3}$ | 2.45 |
| WB746 (proto) | $1.32 \times 10^{-3}$ | $1.49 \times 10^{-3}$ | 1.13 |
| SB120 Trp$^+$-1 (*aroD*) | $1.56 \times 10^{-3}$ | $9.50 \times 10^{-5}$ | 0.06 |
| WB719 (*aroD*) | $1.39 \times 10^{-3}$ | $1.05 \times 10^{-4}$ | 0.07 |

[a] Trp$^+$ transformants were selected on MG agar + SHK (25 μg/ml).

[b] Shk$^+$ transformants were selected on MG agar + tryptophan (25 μg/ml).

**Genetic analysis of the aro mutation in strain RB163.** To test whether, in fact, strain RB163 carried a mutation in the *aroD* gene, the mutant was transformed with limiting quantities of DNA isolated from two independent *aroD* strains, SB120 Trp$^+$-1 and WB719. Transformants were independently selected for SHK independence and tryptophan independence. The frequencies for each marker were then calculated and the Shk$^+$:Trp$^+$ ratios were compared with those derived from the prototrophic crosses. When strain RB163 was transformed with DNA from the two *aroD* strains, the SHK marker, with respect to the *trpC2* locus, was transferred at frequencies of about 0.06 and 0.07 (Table 4). These values are 20 to 30 times lower than the marker frequency ratios derived from the prototrophic crosses. These results indicate a close linkage between the *aroD* locus and the *aro* mutation in strain RB163.

Two other lines of evidence indicate that strain RB163 is an authentic *aroD* mutant. First, the mutant accumulated as much intracellular DAHP as did another *aroD* mutant, SB120, but less than did *aroB* or *aroC* strains (data not shown). Secondly, PBS1-mediated two-factor transduction crosses indicate that the *aro* mutation in strain RB163 cotransduces only with *lys* and at very low frequency (Table 7). No cotransfer was detected with any of 10 other markers distributed along the chromosome. Such low frequency cotransfer with *lys* is characteristic of the *aroD* locus (32).

**Comparison of strain RB163 with other aro mutants.** It was originally observed that an *aro* mutant, SB202 (*aroB*), was capable of synthesizing near normal levels of MK-7 even when grown in the absence of SHK (S. K. Farrand and H. W. Taber, manuscript in preparation). In fact, unlike strain RB163, strain SB202 was phenotypically indistinguishable from strain RB1 with regard to growth, cytochromes, sporulation, and resistance to kanamycin.

Consequently, the phenotypic characteristics of a number of other *aro* mutants, obtained

TABLE 5. *Phenotypic characteristics of SHK-independent transformants of strain RB163*

| Strain | Colony size on TBAB[a] (cm) | Growth stimulation by SHK[b] | Response to kanamycin[c] | Relative cytochrome concn[d] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | *a* | *b* | *c* | $c_1$ | *o* |
| RB163 | 0.1-0.2 | ++++ | Resistant | 1 | 1 | 1 | 5+ | 5+ |
| RB163 Shk$^+$-1 to RB163 Shk$^+$-20 | 1-2 | - | Sensitive | 4 | 5 | 5 | 0 | 0 |
| RB1 | 1-2 | - | Sensitive | 4 | 5 | 5 | 0 | 0 |

[a] Determined as described in Table 2.
[b] TBAB medium was supplemented with SHK at 50 μg/ml.
[c] Determined as described in Table 2.
[d] Determined by microspectroscope examination of dithionite-reduced whole cells as described in Materials and Methods. Values are expressed on a scale of from 0 (absent) to 5 (maximal) based on the intensities of the α-absorption bands for the different heme components.

TABLE 6. *Aromatic amino acid pathway enzyme activities in strains RB1, RB163, and some early aro mutants*

| Strain[a] | Aromatic genotype or phenotype | Specific activity | | | |
|---|---|---|---|---|---|
| | | DAHP synthase[b] | DHQ synthase[c] | DHQ dehydratase[d] | DHS reductase[e] |
| RB1 | Aro$^+$ | 8.12 | 6.35 | 7.66 | 3.10 |
| RB163 | Aro$^-$ | 17.32 | 13.32 | 21.54 | <0.02 |
| SB163A | *aroA* | <0.01 | NA[f] | NA | NA |
| SB167 | *aroB* | NA | <0.03 | NA | NA |
| SB121 | *aroC* | NA | NA | 0.075 | NA |
| SB120 | *aroD* | NA | NA | NA | <0.02 |
| RB163 Shk$^+$-1 | Aro$^+$ | NA | NA | NA | 7.92 |

[a] Strains RB1 and RB163 Shk$^+$-1 were grown in MG medium supplemented with tryptophan (25 μg/ml). All other strains were grown in MG medium supplemented with both tryptophan and SHK (25 μg/ml each).
[b] Expressed as nanomoles of DAHP formed per minute per milligram of protein.
[c] Expressed as nanomoles of DHQ consumed per minute per milligram of protein.
[d] Expressed as nanomoles of DHS formed per minute per milligram of protein.
[e] Expressed as nanomoles of nicotinamide adenine dinucleotide phosphate reduced per minute per milligram of protein.
[f] NA, Not assayed.

from E. Nester, were determined and compared with strains RB1, SB202, and RB163. Such mutants could be divided into two classes (Table 8). Class I, as represented by only three strains (RB163, JH465, and WB2438), exhibited reduced growth and was asporogenous and cytochrome deficient. As with strain RB163, both strains JH465 and WB2438 phenotypically reverted to wild type when grown in the presence of SHK. Class II comprised the vast majority of *aro* mutants. Like strain SB202, they were phenotypically identical to strain RB1 when grown in the absence of SHK (Table 8). SHK had no effect on the characteristics of class II strains.

**Kanamycin sensitivity of class I and class II aro mutants.** Because strain RB163 was originally isolated as a spontaneous kanamycin-resistant mutant, the other two class I strains and several class II mutants were tested for their response to this antibiotic. The results for strain RB1 and the three class I mutants are presented in Fig. 5. Strain RB1, grown in TMG medium, and the class I strains grown in TMG medium supplemented with SHK were all sensitive to kanamycin at 1.5 μg/ml. However, when grown in the absence of SHK, the class I mutants exhibited resistance to the antibiotic.

When grown under conditions in which class I mutants showed antibiotic resistance, class II strains remained sensitive (Fig. 6). However, it should be noted that strain SB163A appeared to show slight resistance, as growth was not affected until almost 30 min after drug addition and was not completely stopped for an additional 90 min.

**MK analysis of class I and class II aro mutants.** The major physiological difference between the class I mutant, RB163, and the class II mutant, SB202, appears to be the inability of the former to synthesize MK-7 when grown in the absence of SHK. Furthermore,

TABLE 7. *Cotransfer values of the aroD locus in strain RB163 with other auxotrophic markers*

| Transducing lysate | Unselected marker[a] | No. of Shk+ transductants tested[b] | No. incorporating second marker | Cotransfer (%) |
|---|---|---|---|---|
| $Mu_6u_5u_{16}$ | *purA16* | | 0 | 0 |
| | *leuA* | 100 | 0 | 0 |
| | *metB* | | 0 | 0 |
| BD71 | *argC* | | 0 | 0 |
| | *pyrA* | 100 | 0 | 0 |
| | *hisA1* | | 0 | 0 |
| BD40 | *argA* | 100 | 0 | 0 |
| | *pheA* | | 0 | 0 |
| GSY240 | *ilvA* | 100 | 0 | 0 |
| GSY189 | *thr* | 100 | 0 | 0 |
| GSY390a | *lys* | 400[c] | 13[c] | 3.2 |

[a] Primary selection was for Shk+ on MG agar + tryptophan supplemented with the auxotrophic requirements of the donor lysate. Amino acids were supplied at 20 μg/ml, purines and pyrimidines at 1 μg/ml.

[b] Tested on complete MG agar lacking only the auxotrophic requirements in question.

[c] Combined results from two separate experiments using the same lysate batch.

strain RB163 became phenotypically indistinguishable from class II strains when grown under conditions allowing MK-7 biosynthesis. To determine whether this correlation between phenotype and MK-7 biosynthesis was general, the other two class I mutants and several class II strains were analyzed for their ability to synthesize quinone when grown in the absence of SHK. Thin-layer chromatograms of the total extractable lipid from membranes of class I and class II strains are shown in Fig. 7 and 8. Although the chromatograms are only semiquantitative, it is clear that, like strain RB163, strains JH465 and WB2438 were unable to synthesize detectable MK-7 unless grown in the presence of SHK (Fig. 7). However, the phenotypically wild-type class II strains synthesized MK even when grown in the absence of the aromatic precursor (Fig. 8). One should note that certain strains, such as SB121 and WB719, appear to synthesize more quinone than do others.

## DISCUSSION

We have described a mutant of *B. subtilis* strain 168 unable to synthesize MK-7 unless

TABLE 8. *Phenotypic characteristics of aro mutants of B. subtilis*

| Class | Strain[a] | Aromatic gene defect | Colony size (cm)[b] | Sporulation (%)[c] | | Relative cytochrome concn[d] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Nutrient sporulation medium | AK medium | *a* | *b* | *c* | $c_1$ | *o* |
| Parental | RB1 | None | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| I | RB163 – SHK | *aroD* | 0.1-0.2 | <0.1 | <0.1 | 1 | 1 | 1 | 5+ | 5+ |
| | + SHK | *aroD* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | JH465 – SHK | *aroB* | 0.1-0.2 | <0.1 | <0.1 | 1 | 1 | 1 | 5+ | 5+ |
| | + SHK | *aroB* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | WB2438 – SHK | *aroG* | 0.1-0.2 | <0.1 | <0.1 | 1 | 1 | 1 | 5+ | 5+ |
| | + SHK | *aroG* | 1-1.5 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| II | SB163A | *aroA* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | SB163B | *aroA* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | SB165 | *aroB* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | SB167 | *aroB* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | SB202 | *aroB* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | SB121 | *aroC* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | SB122 | *aroC* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | SB120 | *aroD* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | WB746 | *aroD* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | WB905 | *aroI* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |
| | WB906 | *aroI* | 1-2 | >50 | >50 | 4 | 5 | 5 | 0 | 0 |

[a] SHK was supplied to class I strains at 25 μg/ml.

[b] Estimated on TBAB after 24 h of growth at 37 C.

[c] Estimated as described in Table 2.

[d] Determined as described in Table 5.

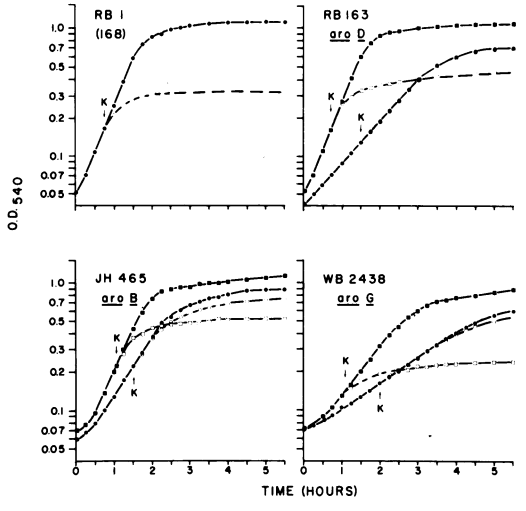


FIG. 5. *Effect of kanamycin on the growth kinetics of strain RB1 and class I aro mutants. Cells were grown in 25 ml of TMG medium. At growth levels indicated by the arrows, each culture was divided into two parts. Kanamycin was added to one half of each culture to a final concentration of 1.5 μg/ml, and growth was followed as described in Materials and Methods. Symbols: ●, unsupplemented TMG medium; ○, TMG medium + kanamycin; ■, TMG medium + SHK (50 μg/ml); □, TMG medium + SHK and kanamycin.*

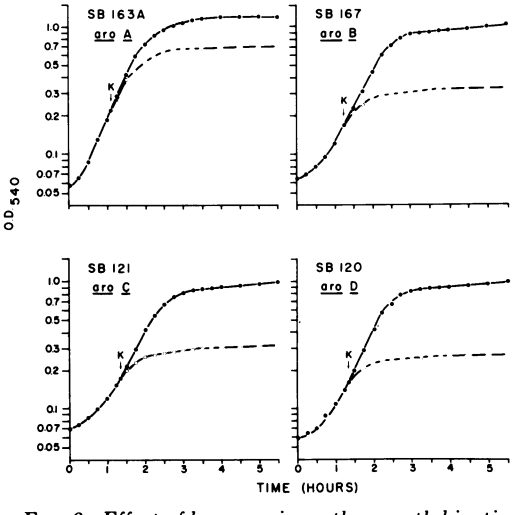


FIG. 6. *Effect of kanamycin on the growth kinetics of class II aro mutants. Experimental conditions were the same as those described in Fig. 5. Symbols: ●, unsupplemented TMG medium; ○, TMG medium + kanamycin.*

grown in the presence of a common aromatic amino acid intermediate such as SHK. Enzyme assays and genetic analysis indicate that the mutant strain, RB163, lacks DHS reductase activity and carries a mutation at the *aroD* locus. Clearly, the *aroD* mutation in strain RB163 abolished the ability of the mutant to synthesize MK-7. In this respect, strain RB163 closely resembles the class I MK-deficient mutants of *Staphylococcus aureus* described by Sǎsǎrman et al. (26). Like these mutants, strain RB163 requires either aromatic end products or SHK for growth but will synthesize MK only when grown in the presence of SHK.

Marjai et al. (19) have also reported on the isolation of poorly growing aromatic amino acid auxotrophs of *B. subtilis* during a search for hemin-requiring mutants. Although no biochemical analyses were reported, these were probably class I mutants.

Interestingly, strain RB163 and the mutants of Sǎsǎrman and of Marjai were all isolated by selecting for resistance to aminoglycoside antibiotics. These small-colony, antibiotic-resistant mutants all have common deficiencies in some components of the electron transport chain. The question arises as to why such

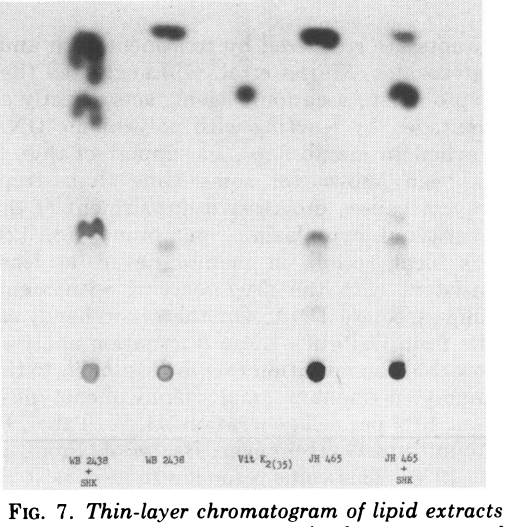


FIG. 7. *Thin-layer chromatogram of lipid extracts from class I aro mutants grown in the presence and absence of SHK. Chromatographic conditions were as described in Fig. 4. From left to right, samples were: total lipid extract from membranes of strain WB2438 (aroG) grown in 500 ml of TMG medium + SHK (50 μg/ml); total lipid extract from membranes of WB2438 grown in 1,000 ml of TMG medium; authentic MK-7 (vitamin $K_2$(35); total lipid extract from membranes of strain JH465 (aroB) grown in 1,000 ml of TMG medium; total lipid extract from strain JH465 grown in 500 ml of TMG medium + SHK. Cells were harvested at $T_1$ and lipids were extracted by method B.*

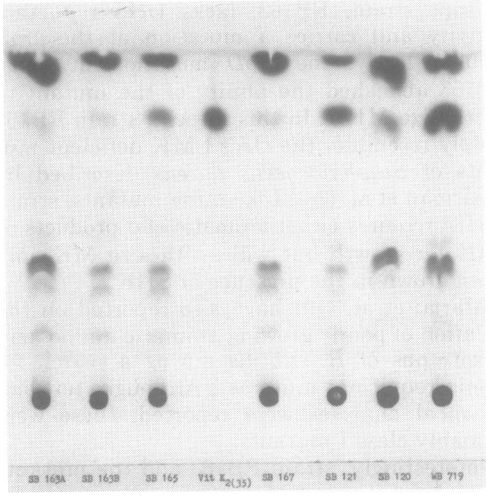


FIG. 8. *Thin-layer chromatogram of lipid extracts from class II aro mutants grown in the absence of SHK. Chromatographic conditions were as described in Fig. 4. Each sample represents the total lipid extract from membranes of cells grown in 500 ml of TMG medium. For aro designations of each strain see Table 1. Cultures were all harvested at $T_1$ and lipids were extracted by method B.*

mutants are recovered by treatment with aminoglycosides. Marjai et al. (19) suggested that streptomycin, a cationic agent, acts directly as a mutagen by reacting with polyanionic DNA attached to membranes. In support of this, it has been known for some time that streptomycin causes progressive impairment of the integrity of cytoplasmic membranes (8, 12). This local action on membranes is at least consistent with the drug reacting with membrane-associate DNA. On the other hand, results from Delbruck-Luria fluctuation analyses show that the mutation rate in *B. subtilis* to the kanamycin-resistant, small-colony phenotype is about $10^{-8}$ per cell generation (H. W. Taber, P. Bitoun, and G. Halfenger, Bacteriol. Proc., p. 139, 1970). This value is too low to be consistent with a mutagenic action for aminoglycoside antibiotics, but instead suggests that the drugs act solely as selective agents.

Why respiratory deficiencies are favored by this selective technique is not understood. However, results from uptake experiments with several kanamycin-resistant mutants of *B. subtilis*, including strain RB163, indicate that they are unable to transport the drug across the cytoplasmic membrane (H. W. Taber and G. Halfenger, manuscript in preparation). It is conceivable that defects in oxidative metabolism and energy production, especially those which involve membrane components, could block active transport of aminoglycoside antibiotics. In support of this is the recent isolation of mutants of *E. coli* lacking membrane-associated $Mg^{2+}$-$Ca^{2+}$-stimulated adenosine triphosphatase activity by selection for resistance to neomycin (15).

If this interpretation is correct, mutants isolated by other techniques which are phenotypically similar to the kanamycin-selected mutants should also be drug resistant. In fact, the two class I *aro* mutants described here (strains JH465 and WB2438) are phenotypically identical to strain RB163 and are also resistant to kanamycin. Strain WB2438 was isolated during a search for regulatory mutants which could grow only on minimal media containing both aromatic supplements and SHK (E. W. Nester, personal communication). Strain JH465 was specifically isolated as a small-colony mutant on nutrient agar after mutagenesis. Neither strain was initially isolated by drug resistance. Moreover, certain cytochrome *a*-deficient mutants of *B. subtilis* studied by Taber and Freese (submitted for publication) which phenotypically resemble strain RB163 are also resistant to aminoglycoside antibiotics.

In addition to poor growth, aromatic supplement requirement, and inability to synthesize MK-7, strain RB163 shows other phenotypic characteristics which differ from the parent strain. Most noticeable is the cytochrome deficiency and the inability to sporulate. Although no revertants have been isolated, we conclude that these phenotypic properties result from the *aroD* block because (i) growth in the presence of SHK will allow complete phenotypic reversion, and (ii) transformation to SHK independence is accompanied by permanent reversion of the pleiotropic characteristics. The nature of the relationship between a block in the aromatic pathway and the cytochrome and sporulation phenotypes will be discussed in a future publication. It is suggested, however, that every phenotypic characteristic exhibited by strain RB163 can be attributed to the inability of the mutant to synthesize MK-7.

The results indicate that the pleiotropic class I aromatic amino acid auxotrophs can be distinguished from those of class II by their inability to synthesize MK-7. Although no activities of the appropriate enzymes could be demonstrated in crude extracts from class II mutants (see Table 6), we infer that these strains differ from those of class I only in the completeness of the metabolic blocks. Thus, class II mutants are bradytrophic, allowing sufficient substrate past the *aro* blocks to satisfy the quinone requirement. In support of this is the observation that

DAHP synthase activity can be demonstrated in the class II *aroA* mutant SB163 if and only if cadmium is added to the reaction mixture (E. W. Nester and W. Nakatsukasa, personal communication).

It is most probable that the procedure commonly used to isolate *aro* mutants actually selects against class I mutations. The standard protocol has been to select for mutants which show a growth requirement on minimal media for the entire aromatic end product pool. These mutants are then tested for their growth response to aromatic intermediates such as SHK. Since the class I mutants grow very poorly on minimal medium supplemented with aromatic end products alone, it is likely that such strains would be lost during the initial selection.

Use of the aminoglycoside selection technique affords the opportunity for isolation of mutants unable to synthesize MK. Such mutants could be used to investigate the biochemistry, genetics, and control of MK biosynthesis, and would also be valuable in the study of MK function in electron transport and in other membrane-localized cellular processes.

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