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Genetic Requirements for Potassium Ion-Dependent Colony Spreading in *Bacillus subtilis*

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Undomesticated strains of *Bacillus subtilis* exhibit extensive colony spreading on certain soft agarose media: first the formation of dendritic clusters of cells, followed by spreading (pellicle-like) growth to cover the entire surface. These phases of colonization are dependent on the level of potassium ion (K^+) but independent of flagella, as verified with a mutant with a *hag* gene replacement; this latter finding highlights the importance of sliding motility in colony spreading. Exploring the K^+ requirement, directed mutagenesis of the higher-affinity K^+ transporter KtrAB, but not the lower-affinity transporter KtrCD, was found to inhibit surface colonization unless sufficient KCl was added. To identify other genes involved in K^+-dependent colony spreading, transposon insertion mutants in wild-type strain 3610 were screened. Disruption of genes for pyrimidine (*pyrB*) or purine (*purD*, *purF*, *purH*, *purL*, *purM*) biosynthetic pathways abolished the K^+-dependent spreading phase. Consistent with a requirement for functional nucleic acid biosynthesis, disruption of purine synthesis with the folic acid antagonist sulfamethoxazole also inhibited spreading. Other transposon insertions disrupted acetoin biosynthesis (the *alsS* gene), acidifying the growth medium, glutamine synthetase (the *glnA* gene), and two surfactin biosynthetic genes (*srfAA*, *srfAB*). This work identified four classes of surface colonization mutants with defective (i) potassium transport, (ii) surfactin formation, (iii) growth rate or yield, or (iv) pH control. Overall, the ability of *B. subtilis* to colonize surfaces by spreading is highly dependent on balanced nucleotide biosynthesis and nutrient assimilation, which require sufficient K^+ ions, as well as growth conditions that promote sliding motility.

It is now known that various wild-type *Bacillus subtilis* strains can form structured multicellular biofilms in the form of floating pellicles on liquid media (3, 4) and pellicle-like surface colonies on semisolid media (18). In the latter case, different phases of colonization can be visualized. In the first phase, cells spread over the surface as dendritic branching colonies. If sufficient potassium ions are present, the cells enter a second growth phase in which the colony can spread over the entire surface as a pellicle-like film. Both phases of surface colonization appear to be independent of flagella and require secretion of surfactin (a lipopeptide with potent surfactant properties [23]). These results are consistent with a sliding motility mechanism, as described by Henrichsen (12), which is defined as surface translocation produced by expansive forces in the growing colony combined with special surface properties to lower the friction between the cells and substrate. However, it is notable that the original observations of sliding motility suggested that a spreading zone of sliding bacteria consists of a single layer of densely packed cells (12). The colony spreading we have observed recently (18) and explore here is not due to expansion of a monolayer of cells but instead represents robust multicellular colony formation akin to that of the pellicles mentioned above.

Although sliding motility is known to occur in a variety of gram-negative and gram-positive bacteria (reviewed by Harshey [10]), surprisingly little is known of the underlying mechanisms of this type of motility. Kolter and coworkers have investigated some aspects of sliding motility in *Mycobacterium smegmatis* and found that effective colony spreading requires the formation of acetylated glycopeptidolipids in the outermost layer of the cell envelope (24, 25). These workers have proposed that the hydrophobic fatty acyl tails of glycopeptidolipids act to lower the friction to facilitate sliding motility on the surface of the solidified medium. Similarly, in undomesticated *B. subtilis* strains surface motility is dependent on the secretion of surfactin, mentioned above, but the link between surfactin and surface colonization has focused primarily on flagellum-dependent swarming (14, 17), not sliding motility.

The main goal of the work presented here was to identify genes required for the K^+-dependent phase of colony spreading. K^+ ions play an essential role in bacterial physiology, including osmoregulation, maintenance of cellular pH, and signaling, and have specific roles in the structure and function of numerous enzymes (reviewed by Epstein [6]). It is known that the *B. subtilis* genome encodes a variety of potential K^+ transporters and channels. Two K^+ transporters are encoded by the KtrAB (higher-affinity) and KtrCD (lower-affinity) systems and may serve a primary role in osmoregulation in response to hypertonicity (13). Of less certain function in K^+ transport is a K^+-stimulated ATPase that is induced by growth at low K^+ concentration (28). We expected that potassium uptake systems would be required to support sufficient levels of intracellular K^+ ions for the K^+-dependent surface colonization, and this was explored by directed mutagenesis. To find other genes required for surface spreading, transposon mutagenesis was conducted in a wild-type *B. subtilis* strain (16), and mutants were screened for lack of colony spreading in the

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TABLE 1. *B. subtilis* strains used in this work

Strain	Genotype/phenotype	Source and/or reference
3610	Undomesticated wild type	NCIB 3610 and reference 3
JH642	*trpC2 pheA1*	J. Hoch
GHB1	Δ(*ktrAB*::Neo)	E. Bremer
GHB6	Δ*ktrC*::Spec	E. Bremer
GHB12	Δ*ktrD*::Tet	E. Bremer
SF62S	*tnrA62*ΩTn*917*Δ*erm*::Spec *trpC2*	S. Fisher
Derivatives of strain 3610		
DS64	Δ*hag*::*mls*	17
DS1010	pIC333	16
MH11	*tnrA62*ΩTn*917*Δ*erm*::Spec	This work
RF3K1	*alsS*ΩTn*3*K1 Spec	This work
RF3K3	*glnA*ΩTn*3*K3 Spec	This work
RF3S3	*srfAB*ΩTn*3*S3 Spec	This work
RF3S7	*srfAB*ΩTn*3*S7 Spec	This work
RF3S11	*srfAA*ΩTn*3*S11 Spec	This work
RFK5	*purD*ΩTnK5 Spec	This work
RFK6	*pyrB*ΩTnK6 Spec	This work
RFK16	*purL*ΩTnK16 Spec	This work
RFK20	*purL*ΩTnK20 Spec	This work
RFK21	*purH*ΩTnK21 Spec	This work
RFK32	*purH*ΩTnK32 Spec	This work
RFK33	*purM*ΩTnK33 Spec	This work
RFK34	*purF*ΩTnK34 Spec	This work
RFKT7	Δ(*ktrAB*::Neo)*1*	This work
RFKT22	Δ*ktrC*::Spec	This work
RFKT31	Δ*ktrD*::Tet	This work
RFKT37	Δ*ktrC*::Spec Δ*ktrD*::Tet	This work

presence of K^+ ions. This work marks the beginning of attempts to define the genetic requirements for K^+-dependent sliding motility and surface colonization by *B. subtilis*.

MATERIALS AND METHODS

Strains and media. *B. subtilis* strains, listed in Table 1, were routinely grown at 37°C on Luria-Bertani (LB) medium containing (per liter) 10 g tryptone, 5 g yeast extract, and 10 g NaCl, supplemented with 1.5% (wt/vol) agar where needed to make plates. Throughout this work, surface colonization phenotypes were detected by growth on a casein digest-mannitol medium (CM) solidified with 0.3% (wt/vol) agarose (Fisher Scientific; low electroendosmosis grade with a gel strength of >1,000 g cm^{-2}) as described in detail elsewhere (7); briefly, it contained (per liter) 10 g D-mannitol, 10 g casein digest (N-Z-Amine A; Sigma-Aldrich Co.), and a trace metal mixture (H medium metals) (7). Use of unbuffered CM agarose medium (initial pH 6.6 to 6.8 in different batches of N-Z-Amine A) gave more discrete dendritic growth, while a medium (CMK) with a K^+-ion source, 7 mM K_2HPO_4 (added separately from a 100× stock; final pH 7.3), produced the most robust surface colonization (7). Here, except for the transposon screening (below), we controlled pH, phosphate, and K^+-ion levels in the CM-type media by addition of either 7 mM Na_2HPO_4 (CMN medium) or 7 mM K_2HPO_4 (CMK medium). To determine the threshold for K^+ ion-dependent colony spreading on CMN agarose, various levels of KCl were added to the medium. In other experiments, these media were supplemented with purine or pyrimidine precursors, acetoin, glutamine, or antibiotics from sterile stocks as indicated in the text. Acetoin was added at 20 mM to mimic the maximal level of acetoin released by wild-type *B. subtilis* strains during assimilation of glucose (29). Soft agarose plates, typically dried 6 to 8 h or overnight in a laminar-flow hood, were inoculated in the center with a sharpened toothpick from regions of uniform growth (overnight, 37°C) on streaked LB agar plates (with antibiotics as necessary); plates dried overnight were usually incubated in a humidified plastic box.

Transposon mutagenesis. For transposon mutagenesis, a temperature-sensitive vector carrying mini-Tn*10*, pIC333, in wild-type strain 3610 was used; this construct, strain DS1010, and conditions for generating transposon libraries have been described in detail (16). Spectinomycin-resistant colonies, grown by plating on LB agar supplemented with 100 μg ml^{-1} spectinomycin followed by overnight growth at 37°C, were replicated with sterile toothpicks onto gridded plates of CM2 (unbuffered) and CMK2 (7 mM K_2HPO_4), which were solidified with twice the level of agarose normally used in the colony-spreading assay (0.6%, wt/vol) to prevent colony overgrowth. After overnight growth at 40°C, the plates were scored and mutants that failed to form a large surface colony (i.e., only showed dendritic growth) were picked and streaked on LB spectinomycin agar. Colonies from these latter plates were inoculated to the center of individual CM and CMK soft agarose (0.3%) plates (in duplicate or triplicate) to confirm the defective K^+ ion-dependent surface colonization phenotype and to LB agar containing 1 μg ml^{-1} erythromycin plus 25 μg ml^{-1} lincomycin (macrolide-lincosamide-streptogramin B resistance from pIC333) (31); colonies with macrolide-lincosamide-streptogramin B resistance were discarded. In analysis of a second transposon insertion library, the CM2 and CMK2 plates (0.6% agarose) were supplemented with 1 mM uridine and 1 mM inosine, as precursors of pyrimidines and purines by their respective salvage pathways (33), and 0.1 mM thiamine, which is derived from the purine biosynthetic pathway.

Transposon insertion sites were identified by cloning into *Escherichia coli* for DNA sequencing as described by Kearns et al. (16). That the transposon and the mutation causing the surface growth defect were inseparable was determined by backcrossing with SPP1-mediated phage transduction for many of the insertions, as described in the text (16).

Construction of other mutant strains. A *B. subtilis* 168 strain (SF62S) containing a Tn*917* insertion in the gene for the TrnA transcription factor was obtained from S. Fisher (Boston University School of Medicine). SF62S, with the genotype *tnrA62*ΩTn*917*Δ*erm*::Spec *trpC2*, was used as a source for SPP1-mediated phage transduction of wild-type strain 3610. Potential transductants were screened for spectinomycin resistance (100 μg ml^{-1}) and sensitivity to erythromycin (5 μg ml^{-1}). Several isolates were then tested for disruption of the *tnrA* gene, using inability to grow with a purine (hypoxanthine) as sole N source (27); this generated strain MH11. For the latter growth medium, defined minimal medium H lacking NH_4Cl (8) with 0.2% glucose, 5 mM hypoxanthine, and 1.5% (wt/vol) agar was used, and the plates were supplemented with spectinomycin (100 μg ml^{-1}). The wild-type strain 3610 grew well on this medium when spectinomycin was deleted.

Strains of 3610 with disruptions in two K^+ uptake systems, KtrAB and KtrCD (13), were constructed from mutants (in the *B. subtilis* laboratory strain JH642) obtained from E. Bremer (Philipps University, Marburg, Germany): GHB1, Δ(*ktrAB*::Neo); GHB6, Δ*ktrC*::Spec; and GHB12, Δ*ktrD*::Tet. Each allele was then transferred to the wild-type strain 3610 by SPP1-mediated phage transduction to obtain the following strains: RFKT7, Δ(*ktrAB*::Neo)*1*; RFKT22, Δ*ktrC*::Spec; RFKT31, Δ*ktrD*::Tet; and RFKT37, Δ*ktrC*::Spec Δ*ktrD*::Tet. Strains

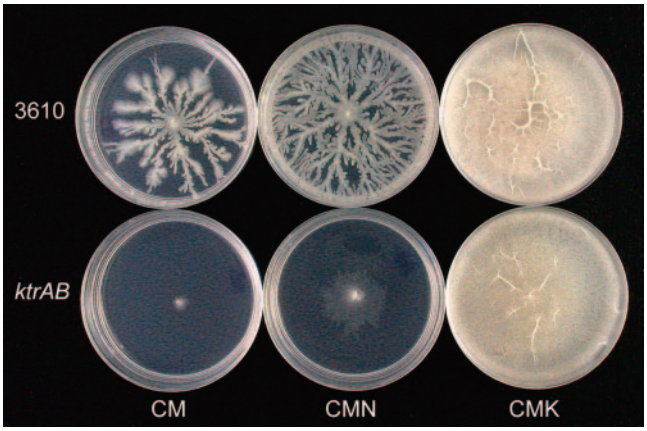

FIG. 1. Surface colonization of *B. subtilis* 3610 and a *ktrAB* mutant on semisolid agarose media is dependent on potassium ions. The casein digest-mannitol agarose media used included CM (low potassium, unbuffered), CMN (CM supplemented with 7 mM Na_2HPO_4), and CMK (CM supplemented with 7 mM K_2HPO_4), as described in Materials and Methods. Plates were inoculated in triplicate and incubated 16 h at 37°C. The plates presented are representative of replicate experiments.

TABLE 2. Summary of *B. subtilis* colony-spreading mutants isolated from strain 3610

Genotype (reason for colony-spreading defect, if any)	Strain	Generation time in CMK broth (min)[a]	Stationary phase OD_{600}[a,d]	Surfactin concn ($\mu g\ ml^{-1}$)[a,b]
Wild type	3610	24	7.24	932
Class I (lack of surfactin)				
srfAB	RF3S3	28	5.28	17*
srfAB	RF3S7	29	6.08	15*
srfAA	RF3S11	33	7.05	15*
Class IIa (reduced growth rate or yield)				
glnA	RF3K3	70	1.46	40
purH	RFK32	83	1.51	308
purL	RFK16	84	1.41	82
purH	RFK21	90	1.55	270
purD	RFK5	116	3.72	420
purM	RFK33	131	1.86	23
purF	RFK34	172	1.39	78
purL	RFK20	188	2.43	109
pyrB	RFK6	253	0.54	6*
	with 1 mM uracil	80	4.79	548
Class IIb (medium acidification and toxicity)				
alsS	RF3K1	38	1.76	6*
Class IIc (potassium transport limitation)				
ktrAB	RFKT7	32[c]	5.78	520

[a] Averages of duplicate determinations in CMK broth determinations.
[b] Surfactin determinations of the cell-free media using authentic surfactin to generate a standard curve by the drop collapse method. These values were determined by dilution with CMK broth, and those with an asterisk were indistinguishable from the background activity. The onset of surfactin release to the medium varied considerably between strains but occurred in early stationary phase. All values, measured in late stationary stage, are averages of duplicate determinations with a variation of <15%.
[c] Growth occurred in CMK broth as it contains 14 mM K^+ (7 mM K_2HPO_4).
[d] OD_{600}, optical density at 600 nm.

were grown in media with the appropriate antibiotics: 10 $\mu g\ ml^{-1}$ neomycin, 10 $\mu g\ ml^{-1}$ tetracycline, or 100 $\mu g\ ml^{-1}$ spectinomycin.

Other methods and chemicals. To verify the phenotypes of surfactin mutants, CMN and CMK plates were supplemented with authentic surfactin (Sigma-Aldrich Co.), added to the surface of plates as described previously (18). Surfactin in the cell-free media, prepared by microcentrifugation of the cells, was assayed by a drop-collapse method (2). In this assay, surfactin was used to generate a standard curve, and CMK broth was used to dilute the samples. Flagellar staining utilized the Ryu stain as described by Heimbrook et al. (11). Motile cells from the edges of surface colonies were allowed to move into 10-μl drops of water for approximately 5 min and then each was stained by this procedure. Acetoin, azaserine, sulfamethoxazole, surfactin, and other antibiotics were obtained from Sigma-Aldrich. Monovalent cations, added as the chloride salts, were analytical grade (>99% purity), except for RbCl, which was listed as having 99.8% purity (Alfa Aesar Co.).

RESULTS

Surface colonization phenotypes of potassium transport mutants. Figure 1 (upper panel) illustrates the K^+ ion-dependent phenotype of the parent wild-type Marburg strain (3610) on semisolid plates of a casein digest-mannitol medium, either unbuffered (CM), buffered with sodium phosphate (CMN), or buffered with potassium phosphate (CMK). As observed previously with another sample of the undomesticated *B. subtilis* Marburg strain (18), the surface colonies were dendritic in the unbuffered and sodium phosphate-buffered media, but if the medium contained at least 1 mM K^+ ions, a profuse colony covered the agarose surface after approximately 12 to 14 h. Consistent with previous observations, *B. subtilis* wild-type cells only rarely exhibit flagella in the spreading phase on CMK media (15), and, using a common flagellar-staining method (10), it was confirmed here that spreading of the 3610 strain is also essentially independent of flagella. In addition, as described in more detail below, profuse colony spreading was also seen in a flagellumless *hag* mutant constructed in the 3610 strain.

As described by Holtmann et al. (13), *B. subtilis* contains at least two K^+ uptake systems, a higher-affinity transporter, KtrAB, and a lower-affinity transporter, KtrCD. Both systems are important for rapid response to changing osmotic stress. To test whether either of these potassium uptake systems was required for K^+-dependent surface spreading, we obtained *B. subtilis* JH642 mutants with disruptions in either *ktrAB*, *ktrC*, or *ktrD* genes (Table 1) and transduced these markers into the wild-type 3610 genetic background. The *ktrAB* mutant was deficient in surface growth unless the media were supplemented with 7 to 8 mM K^+ ions in different experiments (Fig. 1 and data not shown). The KCl requirement could not be replaced by LiCl, NaCl, or CsCl, and only trace growth was seen with RbCl (each at 10 mM). These results support the ideas that the *ktrAB* mutant requires sufficient K^+ to allow both dendritic growth and colony spreading and that the KtrAB transport system is very specific for K^+ ions. In comparison, the *ktrCD* mutant exhibited surface growth phenotypes that were indistinguishable from the parent 3610 strain on CMN and CMK media (data not shown).

Identification of mutants defective in the K^+ ion-dependent, colony-spreading phenotype. In order to identify additional genes required for K^+-dependent colony spreading, we con-

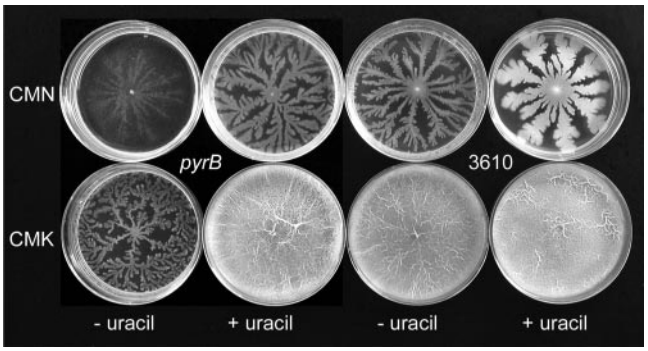

FIG. 2. Typical surface colonization phenotype of a *B. subtilis* transposon mutant selected for lack of response to potassium ions. Shown are surface colonies of the *pyrB* mutant (RFK6; left) or the parent 3610 strain (right) grown on CMN and CMK media with and without uracil (1 mM). CMN (low-potassium) and CMK (high-potassium) agarose media were centrally inoculated and incubated for 16 h at 37°C. The plates presented are representative of replicate experiments; the dendritic growth of the RFK6 mutant on CMN agarose was faint and difficult to photograph, but always visible to the naked eye.

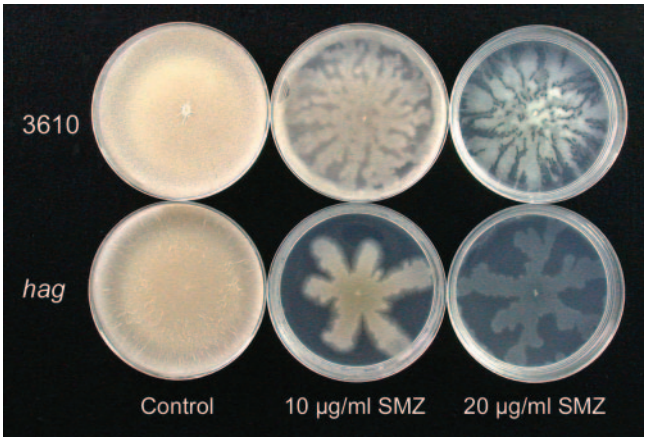

FIG. 3. Effect of the antifolate drug sulfamethoxazole on surface colonization by wild-type *B. subtilis* 3610 and a flagellumless *hag* mutant. CMK plates without and with addition of 10 or 20 μg ml^{-1} SMZ were centrally inoculated and incubated 16 h at 37°C. The plates presented are representative of replicate experiments.

ducted transposon mutagenesis with a temperature-sensitive vector carrying mini-Tn*10*, pIC333, in the 3610 wild-type strain (16). Over 10,000 colonies were screened for the lack of a response to K^+ ions, and eight mutants were obtained and characterized (Table 2). The growth phenotype of a representative mutant (RFK6) from the screen is dendritic growth both in the absence (CMN plates) and presence (CMK plates) of added K^+ ions, but incomplete colonization of the surface (Fig. 2). Incomplete colonization was also seen if the plates were incubated for prolonged periods (24 to 72 h, in a humid atmosphere to prevent drying of the plates). Surprisingly, all eight transposon mutations were localized in genes required for biosynthesis of pyrimidines (*pyrB*) or purines (*purD*, *purF*, *purH*, *purL*, and *purM*). In each case, the mutant phenotype on CMK plates could be bypassed by addition of either uracil or uridine (RFK6, a *pyrB* mutant, as in Fig. 2) or hypoxanthine or inosine (the seven *pur* mutants, data not shown). It is unlikely that the phenotypes of the transposon insertions were due to polar effects on other unrelated genes, as all genes downstream of the insertions were in operons dedicated to either pyrimidine or purine biosynthesis and could be bypassed by addition of pyrimidine or purine precursors, respectively (33).

Sulfamethoxazole, a folate antagonist, inhibits colony spreading to reveal underlying dendritic growth in both wild-type and nonflagellated cells. That purine and pyrimidine biosynthesis mutants were defective in colony spreading suggested that interference with nucleic acid biosynthesis might prevent profuse surface colonization. These considerations led us to attempt to create a purine/pyrimidine imbalance with the antifolate drug sulfamethoxazole (SMZ) (20). SMZ inhibits folic acid biosynthesis and interferes with the biosynthesis of purines, pyrimidines, and certain amino acids (21). As shown in Fig. 3, addition of SMZ at sublethal concentrations (10 or 20 μg ml^{-1}) to CMK agarose medium permitted dendritic growth of strain 3610 but inhibited the profuse surface colonization. At levels of SMZ higher than its MIC in CMK broth (30 μg ml^{-1}), complete inhibition of both dendritic and colony spreading on CMK agarose occurred (not shown). Essentially, while SMZ reduces growth rate and yield (in broth), it is somewhat unique in that it is much more effective in inhibiting the second phase of growth than other antibiotics like ciprofloxacin, kanamycin, tetracycline, or vancomycin and has much less effect on the first (dendritic) phase than these other antibiotics (R. Kinsinger and R. Fall, unpublished).

In addition, we wanted to reexamine the possibility that colony spreading in the wild type is due in part to the presence of flagella that are not readily visualized by standard techniques. For this purpose, a flagellumless mutant with a *hag* gene replacement, constructed in the 3610 genetic background, was examined (17). Spreading motility in the *hag* mutant was essentially identical to that of the wild type and was similarly inhibited by the addition of SMZ (Fig. 3). Examination of spreading cells at the margins of expanding surface colonies of the *hag* mutant by flagellar staining confirmed the lack of flagella. Therefore, these results confirm the importance of purine biosynthesis on spreading over surfaces by a flagellum-independent mechanism.

Analysis of a second Tn library. In order to identify additional genes required for K^+ ion-dependent spreading besides *pur* and *pyr*, we reasoned that we could eliminate these classes of mutations if pyrimidine (uridine) and purine (inosine) precursors were included in the medium. In addition, since the purine biosynthetic pathway also gives rise to thiamine (33), the screening medium was also supplemented with thiamine. From over 6,000 colonies screened, 18 mutants defective in K^+ ion-dependent colony spreading were obtained and analyzed (Table 2). Before gene cloning and analysis of transposon insertion sites, all 18 isolates were tested for correction of the colony-spreading defect by addition of surfactin to the CMN and CMK plates. K^+-dependent spreading motility was rescued by the addition of surfactin for 16 of the isolates, suggesting each contained a disruption of a gene involved in surfactin biosynthesis. Consistent with this prediction, 3 of the 16 surfactin-dependent isolates were determined to contain insertions in two surfactin synthase genes (*srfAA* and two *srfAB* mutations; Table 2). As the phenotypes of these insertions

could be complemented by addition of extracellular surfactin (see below), we conclude that the *srfAA* or *srfAB* mutations were unlikely to have polar effects on unrelated genes.

The two remaining mutants that were not complemented by exogenous surfactin contained transposon insertions in acetolactate synthase (the *alsS* gene) and glutamine synthetase (*glnA*) (Table 1), and both transposon insertions were confirmed to be responsible for the nonspreading phenotype by SPP1-mediated backcrosses. As the *alsS* mutation disrupts formation of the biosynthetic end product acetoin, we attempted to bypass the mutation by the addition of acetoin to the medium. Acetoin addition failed to restore surface colony growth, suggesting that biosynthesis of this compound is not essential for surface-film formation (here and in the rest of the text we refer to surface films as growth that covers the entire surface). Another possible explanation would be that an *alsS* mutation in *B. subtilis* also leads to disruption of cytosolic pH control with release of acids to the medium (29). Thus, it was possible that the spreading defect was a result of growth inhibition by low pH. Compared to the wild type, the *alsS* mutant grew to a lower final cell density and the medium was acidified to pH 5, compared to a final pH of 8 for the wild type. Addition of bis-Tris propane buffer (pH 8.0) was sufficient to restore robust surface spreading to the *alsS* mutant in the presence of K^+ ions (see Fig. 5). It is unlikely that an *alsS* mutation causes significant indirect polar effects, as genes downstream of the insertion are similarly dedicated to acetoin biosynthesis.

To explain the phenotype of the *glnA* mutation, the enzymatic product of the GlnA reaction, glutamine, was added to the medium. In the presence of K^+ ions, addition of glutamine was sufficient to restore robust surface film formation to the *glnA* mutant. It is known that expression of glutamine synthetase (GlnA) is necessary for function of the transcription factor TnrA, which is a global regulator of nitrogen metabolism genes (34). When bound to feedback inhibitors, GlnA binds to TnrA and prevents its DNA from binding to genes regulated by a TnrA box sequence (34, 35). To determine if the surface growth effects seen with the *glnA* mutant were a result of disruption of controls of TnrA, we constructed a *tnrA* mutant strain (MH11) in the 3610 genetic background. When grown on CMN or CMK plates, the MH11 mutant exhibited slower growth but surface growth phenotypes identical to the parent strain (shown in Fig. 1). These results indicate that lack of spreading in the *glnA* mutant is dependent on glutamine biosynthesis but independent of TnrA-mediated gene expression. Polar effects can be ruled out in the case of the *glnA* insertion, as it is the last gene in a two-gene operon (32).

Correlation of mutant phenotypes with surfactin production and growth rates. As the surface growth phenotypes (dendritic and surface film) seen in the transposon mutants might be generated in whole or part by effects on growth rate and/or surfactin secretion, we measured the generation time and surfactin release to the medium for each mutant. The results are summarized in Table 2, and the mutants deficient in K^+-dependent colony spreading are clustered into two general categories. In the class I mutants, comprised of the *srfAA* and *srfAB* mutants, the inability to form dendrites or spread was tightly correlated with a deficiency in surfactin biosynthesis but not growth rate (in CMK broth), compared to the 3610 strain. For this class, the lack of surfactin was sufficient

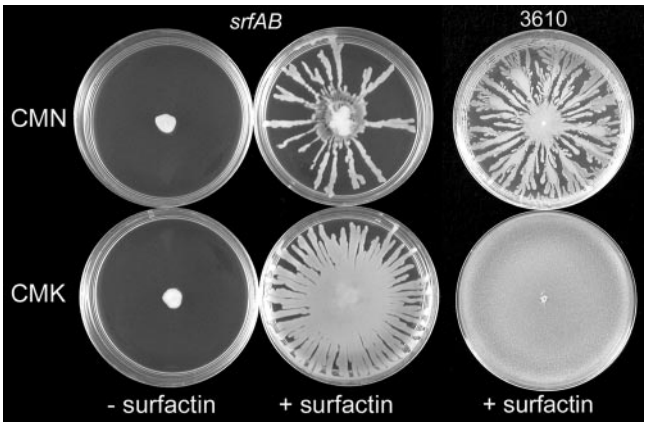

FIG. 4. Surface colonization can be restored to a *srfAB* mutant by the addition of extracellular surfactin. Twenty microliters of surfactin (2 mg/ml) was spotted in the middle of CMN and CMK plates prior to inoculation (with either RF3S3 [*srfAB*] or 3610) and incubation for 16 h at 37°C. The plates presented are representative of replicate experiments.

to explain the colony-spreading defect, as exogenous addition of surfactin partially rescued both dendrite growth and spreading (Fig. 4 and data not shown). It should be noted that in experiments like those shown in Fig. 4, we added a very small amount of surfactin to the center of the plate (40 μg); if the added surfactin diffuses throughout the plate during growth, its final concentration is >100 times lower than would normally be seen in the wild type (Table 2), which might explain the lack of complete rescue by added surfactin.

In the class II mutants, the defect in K^+-dependent spreading was poorly correlated with surfactin production in broth cultures. For example, the *pur* mutants (with the exception of *purM*) produced substantial amounts of surfactin, the *glnA* mutant produced low levels of surfactin, and the *pyrB* and *alsS* mutants produced negligible surfactin in liquid medium. In the cases of the *purM*, *pyrB*, and *alsS* mutants, the defect in surfactin biosynthesis was unable to explain the lack of colony spreading, as these mutants lacked the pellicle-like spreading phase even when surfactin was added to the medium. Furthermore, these mutants must produce some surfactin when grown on surfaces, as each displays the dendritic-spreading phenotype that is surfactin dependent (suggested by the results in Fig. 4). Additionally, when 5-μl drops of water were placed near the edges of expanding dendritic colonies of the *purM*, *pyrB*, and *alsS* mutants, the drops immediately collapsed (in contrast to drops placed in uncolonized areas of the agar surface). This drop collapse is due to reduced surface tension by a surfactant and is indicative of some surfactin secretion during dendritic growth on CMK agarose.

The inability of class II mutants to spread over surfaces appeared to be correlated with growth defects (Table 2), and these mutants could be divided into three subcategories. All of the mutants in class IIa exhibited a 3- to 10-fold increase in generation time and a 2- to 13-fold decrease in the final cell yield when grown in CMK broth. Notably, addition of uracil to the *pyrB* mutant increased the growth rate and yield and rescued K^+-dependent spreading. Similar enhancements of the

growth of several *pur* mutants in CMK broth were seen by addition of 1 mM hypoxanthine, a purine precursor (data not shown). The *alsS* mutant belongs in the growth-defect category (class IIb) because while it grows rapidly in broth, the medium becomes acidified, growth is inhibited, and the final cell yield is reduced. The *ktrAB* mutant also displays a growth defect (class IIc), as it fails to grow unless relatively high concentrations of potassium ions are present. That reduction in growth rate or yield prevents K^+-dependent surface spreading is consistent with the mechanism of sliding motility, a form of surface translocation driven by the expansive properties of a growing population.

The growth defects of the class II mutants can also account for the variability observed in surfactin biosynthesis in broth. Surfactin production in *B. subtilis* is controlled by extracellular accumulation of the ComX and competence-stimulating factor peptides in late-growth-phase cultures (5). Attempts to measure surfactin accumulation at a standard cell density proved intractable. Many of the class II mutants grew to much lower cell densities than the parent strain, as the latter only began to accumulate substantial surfactin (>90 μg ml^{-1}) in CMK broth at cell densities corresponding to optical density values at 600 nm of >4 ($>2.6 \times 10^9$ cells ml^{-1}). For this reason, we followed the growth of each strain until it reached stationary phase, and then analyzed surfactin accumulation in the medium. Presumably the final cell density and concentration of ComX/competence-stimulating factor pheromones are insufficient to induce surfactin biosynthesis in broth, but the same amount of pheromones is sufficient to induce surfactin biosynthesis when the volume is restricted by growth on a surface.

Finally, it should be noted that the rates of extension of surface dendrites of class IIa mutants on CMK agarose plates do not correlate with planktonic growth in CMK broth. For example, comparison of three class IIa mutants (with diverse generation times in broth) including those of *glnA* (70 min), *purM* (131 min), and *pyrB* (253 min) revealed that dendritic growth rates from the point of inoculation were approximately the same (12 to 16 h to reach the edges of the petri dishes). These results highlight the abilities of dendritic structures to grow under conditions of severe nutrient deficiency.

DISCUSSION

Recently, it has become evident that undomesticated *B. subtilis* strains can form highly structured multicellular communities, both as floating biofilms (pellicles) on liquid media (3, 4) and as spreading surface colonies on semisolid media (7, 18). In each case, two phases of growth are evident, where proliferation of highly ordered long chains or colonies of cells is followed by continued growth of the bundled cells into larger, more robust structures. Fruiting body-like structures manifest when sporulation is localized within the context of the pellicle biofilm. A variety of genes required for *B. subtilis* pellicles, biofilms, and fruiting body formation have been identified by directed and nondirected mutagenesis, using various phenotypic tests to visualize gene-specific effects on the formation of these complex multicellular structures (reviewed in references 3, 4, 15, and 30). Notably, none of these genes appears to be related to K^+-ion uptake or homeostasis. Here, we explored the idea that since the two phases of bacterial spreading on agarose media are controlled by the potassium-ion concentration, these conditions could be used to identify additional genes required for pellicle-like colony growth.

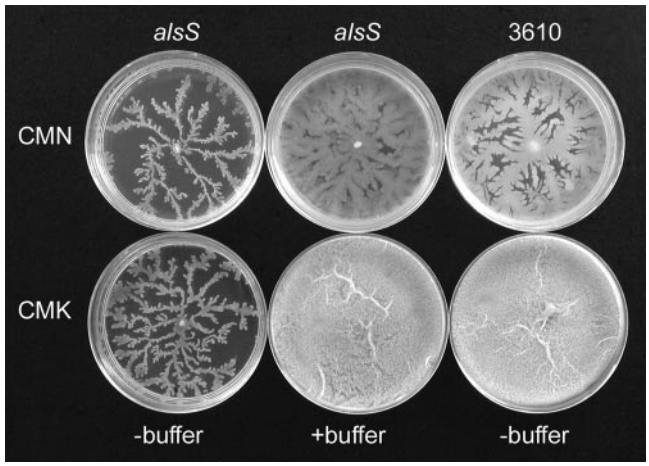

FIG. 5. Surface colonization by *B. subtilis* mutant RF3K1 (*alsS*). RF3K1 (*alsS*) was inoculated in the center of CMN and CMK plates with and without addition of 50 mM bis-Tris propane buffer (pH 8.0), and plates were allowed to grow 18 h at 37°C. For comparison, plates of 3610 from the same experiment (minus bis-Tris propane buffer) are shown; plates of 3610 with this buffer had a similar growth appearance (not shown). The plates presented are representative of replicate experiments.

As mentioned above, K^+ ions play numerous essential roles in bacterial physiology, and various potential K^+ transporters are known to be encoded in the *B. subtilis* genome (6). In this work, we focused on the two major K^+ transporters, the KtrAB (higher-affinity) and KtrCD (lower-affinity) systems, that have been characterized in *B. subtilis* (13). We expected that potassium uptake systems would be required to support sufficient levels of intracellular K^+ ions for the K^+-dependent surface colonization. Consistent with our expectations, disruption of the KtrAB, but not KtrCD, transport system prevented growth and colony spreading on the low-potassium casein digest-mannitol agarose media used (Fig. 1). Addition of sufficient levels of KCl (8 to 10 mM to CMN plates) to the *ktrAB* mutant restored surface growth, likely by use of the lower-affinity KtrCD system. As with the wild-type strain, surface spreading occurs in two phases when high concentrations of potassium are provided to the *ktrAB* mutant. It is reported that *B. subtilis* has normal intracellular levels of K^+ ions of around 350 mM (13), so it is likely that the first phase of growth seen on CM or CMN media in the wild type (with levels of K^+ ions <1 mM) involves the higher-affinity potassium transport system KtrAB, possibly with limited colony spreading due to the K^+ limitation. The role, if any, of the KtrCD transport system in these surface growth phenotypes was not evident in these experiments, as a *ktrCD* mutant was indistinguishable from the parent strain. Perhaps the KtrCD transport system is more important in osmotic stress-induced K^+ transport (13) than in metabolic processes that support surface translocation. The role of other potential K^+ uptake systems in this bacterium also needs to be considered (6).

An unbiased transposon mutagenesis screen was conducted

to find other genes involved in K^+-dependent colony spreading. Surprisingly, many mutations that abolished the spreading phase were localized in genes required for biosynthesis of pyrimidines (*pyrB*) or purines (*purD*, *purF*, *purH*, *purL*, and *purM*). The most likely explanation of this result is that enzymes of both pyrimidine and purine biosynthesis are K^+ ion-dependent, including carbamoyl phosphate synthetase (the first enzyme of pyrimidine biosynthesis) (19) and AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside) transformylase-IMP cyclohydrolase, the last enzyme complex of purine biosynthesis (33), which is very likely to require K^+ ions. This latter conclusion is based on the finding of an essential bound K^+ ion in the crystal structure of avian AICAR transformylase-IMP cyclohydrolase (9) and the finding that the *B. subtilis* gene for this enzyme encodes a highly conserved K^+ ion binding sequence (22). It is notable that all of the *pyr* and *pur* mutants obtained here still required K^+-ion supplementation for complete surface colonization. Colony spreading on CMK medium might be enhanced by the higher levels of K^+ ions (14 mM) added, which might in turn promote normal cellular levels of K^+ ions and support balanced nucleotide biosynthesis or other aspects of cellular metabolism. The phenotype of the *glnA* mutation is likely to be related to an imbalance in pyrimidine and purine biosynthesis, as the glutamine side chain donates N atoms to both pyrimidine and purine rings. The finding that an *alsS* mutant could also produce a similar surface colonization defect was initially surprising. Acetolactate synthase is involved in postexponential-phase production of acetoin from pyruvate (in liquid media) (26). However, we found that the *alsS* mutant rapidly acidified the CM and CMK media used here and, thus, it is likely that this defect simply disrupted normal surface growth by such acidification. The *alsS* gene defect in surface growth was readily overcome by inclusion of a suitable buffer in the medium (Fig. 5).

In toto, the mutant analysis suggests that two factors are important for K^+-dependent surface spreading: surfactin production and rapid growth. We suggest that the cells require a high concentration of intracellular potassium to support balanced nucleotide biosynthesis for RNA and DNA synthesis and the efficient nutrient assimilation that is necessary for rapid cell division. A high growth yield is also important to attain a sufficient cell density to activate surfactin biosynthesis by quorum signaling molecules. Finally, surfactin is secreted to reduce surface tension and allow spreading, which is driven passively by the expansive forces of colony growth. In this regard, dendritic spreading and K^+-dependent spreading can be considered two different manifestations of sliding motility that are perhaps differentiated based on two different thresholds of potassium-ion concentration.

It is likely that K^+-dependent colony spreading is related to the process of pellicle formation (3); as mentioned above, both appear to be two-phase processes initiated by the clustering of cells before a surface colony assembles. Numerous genes necessary for pellicle formation in *B. subtilis* have been identified (4), and we are currently investigating whether pellicle mutants affect the first or second phase of surface colony spreading in *B. subtilis*. Finally, as emphasized by Branda et al. (4), surface colonization and pellicle formation by *B. subtilis* are models for biofilm formation in a gram-positive species. Given recent evidence that wild-type *B. subtilis* strains are common on plant roots (7) and can form stable biofilms on cultured *Arabidopsis* roots and also protect such roots from infection by *Pseudomonas syringae* (1), it seems likely that future work may reveal new aspects of the importance of *B. subtilis* surface colonization in the rhizosphere and the role(s) of K^+ ions and K^+-ion transport in these processes.

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