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New inhibitors of colony spreading in *Bacillus subtilis* and *Bacillus anthracis*

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Abstract

We have recently characterized sliding motility in *Bacillus subtilis* strains that lack functional flagella, and here describe the discovery of inhibitors of colony spreading in these strains as well as the aflagellate pathogen, *B. anthracis*. Aflagellate *B. subtilis* strains were used to screen for new types of antibacterials that might inhibit colony spreading on semi-solid media. From a diverse set of organic structures, *p*-nitrophenylglycerol (NPG), an agent used primarily in clinical laboratories to control *Proteus* swarming, was found to inhibit colony spreading. The four stereoisomers of NPG were synthesized and tested, and only the 1*R*,2*S*- (1*R*-*anti*) and 1*R*,2*R*- (1*R*-*syn*) NPG isomers had significant activity in a quantitative colony-spreading assay. Twenty-six NPG analogs and related structures were synthesized and tested to identify more active inhibitors. *p*-Methylsulfonylphenylglycerol (*p*-SPG), but not its *ortho* or *meta* analogs, was found to be the most effective of these compounds, and synthesis and testing of all four *p*-SPG stereoisomers showed that the 1*R*-*anti*-isomer was the most active with an average IC₅₀ of 16 μM (3 to 5 μg mL⁻¹). For *B. anthracis*, the colony-spreading IC₅₀ values for 1*R*-*anti*-SPG and 1*R*-*anti*-NPG are 12 μM (2–4 μg mL⁻¹) and >150 μM, respectively. For both *Bacillus* species tested, 1*R*-*anti*-SPG inhibits colony spreading of surface cultures on agar plates, but is not bacteriostatic or bacteriocidal in liquid cultures. Work is in progress to find the cellular target(s) of the NPG/SPG class of compounds, since this could lead to an understanding of the mechanism(s) of colony spreading as well as design and development of more potent inhibitors for the control of *B. anthracis* surface cultures.

Keywords

Bacillus subtilis; *Bacillus anthracis*; Colony spreading; *p*-nitrophenylglycerol (NPG); *p*-methylsulfonylphenylglycerol (*p*-SPG)

Bacteria use a variety of motility mechanisms to colonize surfaces. These include swimming and swarming, both dependent on flagella for motility, and twitching, gliding and sliding,

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Supplementary Data

Supplementary data associated with this article (detailed bacterial colony spreading assays, synthesis and characterization of all SPG and NPG isomers) can be found, in the online version, at doi:_____.

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more diverse flagella-independent mechanisms of motility (reviewed by Harsheyⁱ). We have been interested in sliding motility in aflagellate *Bacillus subtilis* strainsⁱⁱ as this type of multicellular movement on laboratory media can exhibit an unusual tendril-like growth mode that precedes extensive web-like colony formation. We have suggested that the formation of tendrils could be a growth strategy for nutrient-deprived cells to colonize natural surfaces such as plant roots that contain large populations of *Bacillus* species.ⁱⁱⁱ When sufficient nutrients are available, the tendrils then spread and form an entire colony or biofilm.

Notably, Henrichsen defined sliding motility as flagella-independent surface translocation driven by expansive forces in the growing colony combined with some type of cell-surface mechanism to lower the friction between the cells and substrate.^{iv} Henrichsen demonstrated this type of motility with *Bacillus anthracis* (10 strains were examined), as well as with many other Gram-negative and Gram-positive bacteria. In the case of *B. subtilis* wild type strains, biosurfactants such as lipopeptides termed surfactins,^v are secreted from the cells. We have shown that disruption of the *srfAA* gene of *B. subtilis* prevents surfactin production and that the mutant does not produce tendrils or extensive films;^{vi} these growth phenotypes are rescued by addition of surfactin. These results confirm the essential role of extracellular surfactants in sliding motility in this species, as proposed by Henrichsen.

Interestingly, *B. anthracis* not only lacks flagellar motility,⁴ but there apparently have been no reports of biosurfactant properties of its cells, capsule surfaces or extracellular products. This raises the question of how this bacillus species lowers the surface tension of the substrate to allow sliding motility. In addition, Henrichsen⁴ observed that sliding motility in *B. anthracis* and other bacteria occurs as a monolayer of cells. This is clearly not the case with the sliding motility we have studied in *B. subtilis* as the tendrils and surface colonies are multicellular.² A similar observation has been discussed by Kaito and Sekimizu^{vii} in their studies of flagella-independent colony spreading in *Staphylococcus aureus* strains. Recently, Stewart et al.^{viii} discovered a type of sliding motility in the Gram-negative species, *Legionella pneumophila*, and demonstrated that this motility is dependent on type II protein secretion. This suggests that a secreted *L. pneumophila* protein may lower surface tension to allow colony expansion. At present, the exact mechanism of sliding motility in *Bacillus* species is uncertain.

In the course of studying the tendril to surface colony transformation of *B. subtilis*, we searched for various inhibitors of this type of colony expansion. We reasoned that such inhibitors might be colony-spreading or biofilm inhibitors. There are relatively few known small molecule biofilm inhibitors in Gram-positive bacteria,^{ix} and apparently few reports of colony-spreading inhibitors. An exception is described by Ogasa et al.,^x where the widely used tetrazolium dye, 2,3,5-triphenyltetrazolium chloride (TTC), can prevent colony overgrowth in enumeration of food and clinical microbial species.

In this article, we describe experiments that take advantage of flagella-less mutants of *B. subtilis* with a *hag* gene disruption in an undomesticated parent as described previously^{2,xi}. Lacking flagella, these *hag* mutants can, apparently, only colonize surfaces by the sliding mechanism described above. As described herein, we have found a commercially available small molecule inhibitor of colony spreading in *hag* mutants, and have used it as the lead molecule to synthesize a family of related compounds, and identified a more potent inhibitor of *Bacillus* surface colony spreading. This family of compounds also inhibits the colony spreading of *B. anthracis* Sterne, an avirulent strain derived from a pathogenic anthrax-causing parent.^{xii}

Few colony-spreading inhibitors have been described in the literature, so we tested dozens of compounds for inhibition of surface colony spreading by a *B. subtilis* *hag* gene mutant (DS64). Examples of its colony spreading phenotypes are shown in Fig. 1. The MK agarose plates used represent a semi-solid medium with complete, defined nutrients,² and when inoculated with the *hag* mutant in the center and incubated overnight (37 °C), a robust surface colony forms (Fig. 1, left). However, when the plates contained relatively high levels of commercial nitrophenylglycerol (NPG; 75 $\mu\text{g mL}^{-1}$), only surface tendrils form (Fig. 1, right). NPG has long been used to inhibit the flagella-dependent swarming of *Proteus* strains (reviewed by Williams^{xiii}), and it is thought to act by disruption of a signal cascade in these Gram-negative bacteria, also including *Serratia marcescens*.^{xiv} It was a surprise that a flagellar swarming inhibitor for Gram-negative bacteria would inhibit flagella-independent colony-spreading in a Gram-positive bacterium.

This effect of NPG on *Bacillus* growth on the MK agarose medium was not due to growth inhibition, as measured in MK broth, or reduction of surfactin release, as measured by a water drop collapse method where 5 μL water drops are placed near the edges of growing colonies or tendrils.⁶ Surfactin, a potent lipopeptide surfactant, is needed for sliding motility in *B. subtilis* as mentioned above.

The stereochemistry of the commercial sample of NPG used in the experiment shown in Fig. 1 was not indicated by the supplier, but upon analysis by NMR and chiral HPLC (Chiralcell® OD column), it was found to be a racemic mixture of the *anti* diastereomer (Fig. 2, compounds **2** and **3**). We wished to determine if the activity of NPG is stereospecific, and therefore synthesized the four possible isomers by the routes shown in Schemes 1 and 2. The synthesis of the two *syn* enantiomers began with aldehyde **4**, which was subjected to the List-modification of the Knoevenagel condensation^{xv} to provide α,β -unsaturated ester **5**. Reduction to the corresponding allylic alcohol (**6**) was accomplished with DIBAL-H, and dihydroxylation with either AD-mix- α or AD-mix- β provided the individual *syn* enantiomers, 1*S*,2*S*-NPG (1*S*-*syn*-, **7**) and 1*R*,2*R*-NPG (1*R*-*syn*-, **8**), respectively, in high enantiomeric excess.^{xvi}

The 1*R*,2*S* (1*R*-*anti*-) and 1*S*,2*R* (1*S*-*anti*-) NPG isomers **2** and **3** were also prepared starting with aldehyde **4** which was treated with vinylmagnesium bromide to provide the corresponding allylic alcohol and then subjected to Sharpless kinetic resolution^{xvii} using either L- or D-diisopropyl tartrate to provide the *R*- and *S*-allylic alcohols, **9** and **10**, respectively, in high enantiomeric excess. The *R*-allylic alcohol (**9**) was then subjected to Sharpless asymmetric epoxidation using D-diisopropyl tartrate to provide epoxide **11** in high enantiomeric excess. Similarly, subjection of *S*-allylic alcohol **10** to Sharpless asymmetric epoxidation using L-diisopropyl tartrate provided epoxide **12** in high enantiomeric excess. Epoxide opening of **11** and **12** with water under acidic conditions occurred at the terminal carbon to provide 1*R*-*anti*-NPG (**2**) and 1*S*-*anti*-NPG (**3**). Related epoxide openings have been studied by Jäger et al.^{xviii} and found to also occur at the terminal position. These general schemes were also used to synthesize analogs of NPG starting with different aldehydes as described in the Supplementary Material.

All 4 NPG isomers were tested on MK agarose plates like those shown in Figure 1. Each isomer was tested at 75 $\mu\text{g mL}^{-1}$ as shown in Fig. 3. In this experiment we used a marker-less *hag* mutant (DS1677), constructed to eliminate possible polar effects of the *hag* strain with an MLS antibiotic marker inserted in strain DS64. As seen in Fig. 3 left, the DS1677 strain produced web-like colonies indistinguishable from those produced by DS64 (Fig. 1). Under these conditions, only the 1*R*-*anti*-NPG (**2**) showed substantial inhibition of surface colony formation (Fig. 3) and as in the case of commercial NPG, surface tendrils (but not colony formation) are observed. No surface colony spreading inhibition was evident with the

1*S-anti*- or 1*S-syn*-NPG isomers (**3** or **7**), and these plates showed the same behavior as the control with web-like surface colony formation being observed. Weak activity was visible with 1*R-syn*-NPG (**8**), where a much thinner, but entire surface colony was observed with no distinct tendrils.

Having the four NPG stereoisomers also allowed us to assess the stereospecificity of NPG inhibition of swarming in *Proteus*. Using the plate swarm assay described in the Supplementary Material, only the 1*R-anti*- (**2**) and 1*R-syn*-NPG (**8**) isomers had activity; at the levels tested ($75 \mu\text{g mL}^{-1}$ in the plate) there was no visible effect of the 1*S-anti*- (**3**) or 1*S-syn*-NPG (**7**) isomers (data not shown).

Notably, in the type of colony growth inhibition assays shown in Fig. 1 and 3, it is not feasible to calculate IC_{50} values. That is, it is difficult to find the inhibitory transition point between tendril and colony growth. To calculate IC_{50} values for the NPG isomers (and other analogs), we employed a colony-spreading assay (S1) based on that used by Kaito and Sekimizu⁷ in studies with *Staphylococcus aureus*. In this assay, a bacterial inoculum of *hag* 1677 is placed at the center of a suitable soft agar medium, and after a defined period of incubation the diameter of the colony is measured. We found that a medium of Tryptic soy broth solidified with agar (7 g L^{-1}) gave the most reproducible results. Typical results are shown in Fig. 4. Each colony's average diameter was measured and the results of duplicate determinations for the NPG stereoisomers are summarized in Table 1. Again, the only NPG isomers with significant activity were 1*R-anti*- and 1*R-syn*- (**2** and **8**), with colony spreading inhibition of 25% and 16%, respectively at $30 \mu\text{g mL}^{-1}$ in the plates. For 1*R-anti*-NPG (**2**), its IC_{50} for inhibition of colony-spreading was determined to be approximately 122 – 132 μM ($25\text{--}27 \mu\text{g mL}^{-1}$ in different independent experiments, each performed in duplicate). The stereochemistry of the glycerol side chain is obviously important in the interaction of the NPG stereoisomers with the cellular target, with the 1*R*, 2*R/S* stereochemistry presumably providing the best binding to this unknown target.

With 1*R-anti*-NPG established as a lead compound, we wished to determine its pharmacophore and develop more potent inhibitors. Specifically, we studied the role of the nitro group and determined whether other polar groups on the aromatic ring could be used in its place. In addition, we probed the effect of locating the polar group at the *meta*-, or *ortho*-positions, and determined whether the hydroxyl groups are required for activity. Our results are shown in Table 1.

We found that substitution of the *para*-nitro group of compound **2** for a *para*-methyl sulfonyl group (SPG, compound **13**) provided a significantly more active compound (95% inhibition with $15 \mu\text{g mL}^{-1}$ ($60 \mu\text{M}$) vs 25% inhibition with $30 \mu\text{g mL}^{-1}$ ($140 \mu\text{M}$), compare Table 1, entries 1 and 5). We next studied the effect of varying the stereochemistry of the hydroxyl groups in the SPG series. Consistent with our findings in the NPG series, the 1*R-anti* substrate (**13**) was the most active, while the 1*R-syn* substrate (**14**) was second most active (Table 1, entries 5 and 6). The 1*S-anti*- (**15**) and 1*S-syn*- (**16**) compounds were essentially inactive (Table 1, entries 7 and 8).

Substitution of the *para*-nitro group with other polar substituents including hydroxy (**17**), and mesyloxy (**18**), provided compounds of comparable or slightly reduced activity as compared to NPG (Table 1, entries 9 and 10; these substrates were prepared as mixtures of all four stereoisomers). Other polar groups at the *para* position, such as carbomethoxy (**19**), and thiomethyl (**20**), provided compounds that were not active (Table 1, entries 11 and 12; these substrates were also prepared as mixtures of all four stereoisomers). Surprisingly, the *para*-sulfonamide substituted compound (**21**) was essentially inactive, while the parent

compound bearing a proton in place of the nitro group was of comparable activity to NPG (Table 1, entries 13 and 14; these substrates were prepared as mixtures of isomers).

Variation of the position of the sulfone was then studied and the corresponding *meta*- and *ortho*- methylsulfones were prepared as mixtures of all four stereoisomers. The *meta*-substituted compound (**23**) showed significantly lower activity (10.5% inhibition), while the *ortho*-substituted compound (**24**) was inactive (Table 1, entries 15 and 16).

We then studied the effect of variation of the hydroxyl groups in the 1*R*-*anti*-SPG series, and found that the presence of the hydroxyl groups provided the most active compounds. Thus, the 1*R*-vinyl alcohol substrate (**25**) that lacks hydroxyl groups at the 2- and 3- positions while active, provided significantly reduced inhibition (Table 1, entry 17, 21% inhibition), while methylation of any or all of the hydroxyl groups provided compounds of low or no activity (Table 1, entries 18 – 21). These results indicate that the optimal compounds in this series bear a *para*-methylsulfone substituent on the aromatic ring, and hydroxyl groups on the aliphatic chain. The stereochemistry of the hydroxyl groups is important for activity, especially at the 1-position where the *R*-configuration is required while at the 2-position the *S*-configuration is favored.

As mentioned above, *B. anthracis* can colonize surfaces of laboratory media by an unknown flagella-independent sliding mechanism. With the results obtained with new colony-spreading inhibitors of *B. subtilis*, it was logical to test them for colony-spreading of *B. anthracis*; we used an avirulent *B. anthracis* strain¹² for these experiments (Table S1 in the Supplementary Material). As with *B. subtilis*, we tried to use a colony-spreading assay based on Tryptic soy broth and soft agar. When the agar level was reduced from 0.7% w/v, as used with *B. subtilis*, to 0.35% w/v and *B. anthracis* Sterne cells were applied to relatively wet plates, an entire giant colony formed overnight, analogous to that seen in *S. aureus* and *S. xylosum*⁷. As shown in Fig. 5, when *B. anthracis* Sterne colony growth was challenged with discs with each of the four SPG stereoisomers, as with *B. subtilis*, the 1*R*-*anti*- and 1*R*-*syn*-SPG isomers exhibited the greatest activity.

For *B. anthracis*, the colony-spreading IC₅₀ values for 1*R*-*anti*-SPG and 1*R*-*anti*-NPG were found to be 12 μ M (2–4 μ g mL⁻¹) and >150 μ M, respectively. Thus, as with *B. subtilis* colony spreading, the 1*R*-*anti*-SPG is also much more effective than 1*R*-*anti*-NPG.

It is tempting to speculate that this work may have led to the discovery of a new type of compound for the treatment of bacterial infection: colony-spreading inhibitors. As summarized in the introduction, the phenomenon of colony spreading on laboratory media is widespread in bacteria, and conceivably could be related to pathogenic infections of tissues. However, there is a dearth of literature on this point. Ironically, the lead inhibitor was discovered by accident as a swarming inhibitor of certain Gram-negative bacteria. Swarming is a multiflagellar motility phenotype, while the colony spreading described here in *B. subtilis* *hag* strains and *B. anthracis* is independent of the presence of flagella. When the cellular target(s) of colony-spreading inhibitors are discovered it may be possible to understand the relationship of swarming and colony spreading inhibition by the NPG/SPG classes of compounds. In the meantime, we have succeeded in synthesizing stereospecific analogs of NPG, including one, *para*-1*R*-*anti*-SPG (**13**), that is a much more effective inhibitor of multicellular colony expansion on plates. It is interesting that 1*R*-*anti*-SPG inhibits colony spreading on solidified media but is not bacteriostatic or bacteriocidal in liquid media. This is true for both *B. subtilis* and *B. anthracis*. This strongly suggests that 1*R*-*anti*-SPG is acting to affect the expression and/or function of some surface structure essential for colony spreading. An important future goal is to discover the identity and role of this surface structure.

Supplementary Material

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Acknowledgments

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Figure 1.

The film spreading of *B. subtilis* hag mutant (DS64; Table S1 in the Supplementary Material), but not tendrils formation, is inhibited by NPG (**1**). MK agarose plates with (right) and without (left) 75 µg mL⁻¹ NPG (**1**) were inoculated in the center with the hag strain and grown for 24 h at 37 °C. These results are typical of triplicate determinations. Note that tendrils growth precedes film formation with and without NPG.

Figure 2.
Stereochemistry of commercial NPG

Figure 3.

1*R*-*anti*-NPG, but not the 1*S*-*syn* or 1*S*-*anti* stereoisomers, inhibits surface colony formation of a *B. subtilis* hag mutant (DS1677; Table S1 in the Supplementary Material). The experiment was performed in triplicate as described in Fig. 1 using MK agarose plates with $75 \mu\text{g mL}^{-1}$ of each compound in DMSO, including commercial NPG (Acros, Inc.). Control plates contained $75 \mu\text{g mL}^{-1}$ of DMSO, the solvent used for each of the NPGs.

Figure 4.

An example of the colony-spreading assay using a *B. subtilis* hag deletion strain (Table S1 in the Supplementary Material). Three microliters of an overnight LB culture of hag DS1677 was spotted on TSB agar that had been dried for 3–4 h. The plates (prepared in duplicate) contained the indicated amounts of 1R-anti-SPG, and were allowed to grow at 32 °C for 16 h. Measurement of colony diameters allowed determination of the IC₅₀-- in this case 3–5 $\mu\text{g mL}^{-1}$.

Figure 5.

1*R*-anti-SPG is an inhibitor of colony spreading in *Bacillus anthracis* Sterne. Of the four SPG stereoisomers, each added at 75 µg per disc, the 1*R*-anti-SPG isomer is the most potent while the 1*R*-syn- isomer is still active, though less potent. The colony-spreading plates shown are Tryptic soy broth soft agar, described in the text, and were inoculated in the center (white "X") with 3 µL of a log phase *B. anthracis* Sterne culture and grown overnight at 37 °C.

Scheme 1.

Synthesis of 1S,2S (1S-syn-) and 1R,2R (1R-syn-) NPG a) 2-(methoxycarbonyl)acetic acid, DMAP, piperidine, DMF; b) DIBAL-H, Et₂O; c) AD-mix- α , 1:1 *t*-BuOH/water; d) AD-mix- β , 1:1 *t*-BuOH/water.

Scheme 2.

Synthesis of 1*S*,2*R*- (1*S*-anti-) and 1*R*,2*S*- (1*R*-anti-) NPG a) vinylmagnesium bromide, THF; b) Ti(i-PrO)₄, D-diisopropyl tartrate, *t*-BuOOH, CH₂Cl₂; c) Ti(i-PrO)₄, L-diisopropyl tartrate, *t*-BuOOH, CH₂Cl₂; d) H₂O, acetic acid.

Table 1

Screening of NPG and SPG analogs for inhibition of colony spreading of a *B. subtilis* *hag* mutant (DS1677^a)

Entry	compound	% Inhibition of colony spreading ^{b,c}
1		24.8
2		16.1
3		1.5
4		0
5		95.3
6		69.1

Entry	compound	% Inhibition of colony spreading ^{b,c}
7	15	1.6
8	16	0
9	17^d	12.9
10	18^d	11.5
11	19^d	0
12	20^d	0
13	21^d	4.5

Entry	compound	% Inhibition of colony spreading ^{b,c}
14	22	12.9
15	23	10.5
16	24	0
17	25	21.3
18	26	10.1
19	27	0

Entry	compound	% Inhibition of colony spreading ^{b,c}
20	<p style="text-align: center;">28</p>	18.5
21	<p style="text-align: center;">29</p>	0
22	DMSO control	0

^a Results from colony expansion experiments like those in Fig. 3, where each compound was tested in duplicate or triplicate (S1). Where replicates varied by more than 10% in average colony diameter, the assay for that compound was repeated.

^b NPG isomers were added at a concentration of 30 $\mu\text{g mL}^{-1}$.

^c All other compounds were added at concentration of 15 $\mu\text{g mL}^{-1}$.

^d Mixture of 4 stereoisomers