D-Amino Acids Trigger Biofilm Disassembly

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Abstract

Bacteria form communities known as biofilms, which disassemble over time. Here we found that prior to biofilm disassembly *Bacillus subtilis* produced a factor that prevented biofilm formation and could break down existing biofilms. The factor was shown to be a mixture of D-leucine, D-methionine, D-tyrosine and D-tryptophan that could act at nanomolar concentrations. D-amino acid treatment caused the release of amyloid fibers that linked cells in the biofilm together. Mutants able to form biofilms in the presence of D-amino acids contained alterations in a protein (YqxM) required for the formation and anchoring of the fibers to the cell. D-amino acids also prevented biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. D-amino acids are produced by many bacteria and thus may be a widespread signal for biofilm disassembly.

Most bacteria form multicellular communities known as biofilms in which cells are protected from environmental insults (1–2). However, as biofilms age, nutrients become limiting, waste products accumulate, and it is advantageous for the biofilm-associated bacteria to return to a planktonic existence (2). Thus, biofilms have a finite life time, characterized by eventual disassembly. *Bacillus subtilis* forms communities on semi-solid surfaces and thick pellicles at the air-liquid interface of standing cultures (1,3–5). Cells in the biofilms are held together by an extracellular matrix consisting of exopolysaccharide and amyloid fibers composed of TasA (5–7). The exopolysaccharide is produced by the *epsA-O* operon and the TasA protein is encoded by the *yqxM-sipW-tasA* operon (8).

After three days of incubation in biofilm-inducing medium, *B. subtilis* formed thick pellicles at the air/liquid interface of standing cultures (Fig. 1A). Upon incubation for an additional three to five days, however, the pellicle lost its integrity (Fig. 1B). To investigate whether mature biofilms produce a factor that triggers biofilm disassembly, we asked whether conditioned medium would prevent pellicle formation when added to fresh medium. Medium from an eight-day-old culture was applied to a C18 Sep Pak column, and concentrated eluate from the column was added to a freshly inoculated culture. The eluate was sufficient to prevent pellicle formation (Fig. 1C). Concentrated eluate from a three-day-old culture had little effect on pellicle formation (Fig. 1D). Further purification of the factor was achieved by eluting the cartridge step-wise with methanol. Elution with 40% methanol resulted in a fraction that was active in inhibiting pellicle formation (Fig. 1E), but had little

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effect on cell growth (Fig. S1). The activity was resistant to heating at 100 °C for 2 hours and proteinase K treatment (Fig. 1F).

Bacteria produce D-amino acids in stationary phase (9). We asked whether the biofilm-inhibiting factor was composed of one or more D-amino acids. Indeed, D-tyrosine, D-leucine, D-tryptophan, and D-methionine were active in inhibiting biofilm formation both in liquid and on solid medium (Figures 1G–H, Figures S2–3). In contrast, the corresponding L-isomers and D-isomers of other amino acids, such as D-alanine and D-phenylalanine, were inert in our biofilm-inhibition assay. Next, we determined the minimum concentration needed to prevent biofilm formation. Individual D-amino acids varied in their activity, with D-tyrosine being more effective (3 µM) than D-methionine (2 mM), D-tryptophan (5 mM) or D-leucine (8.5 mM). A mixture of all four D-amino acids was particularly potent, with a minimum inhibitory concentration of ~10 nM. Thus, D-amino acids could act synergistically. The D-amino acids not only prevented biofilm formation, but also disrupted existing biofilms. The addition of D-tyrosine or a mixture of the four D-amino acids (but not the corresponding L-amino acids) caused pellicle breakdown (Fig. 2A).

D-amino acids are generated by racemases (10). Genetic evidence consistent with the idea that the biofilm-inhibiting factor is D-amino acids came from mutants of *ylmE* and *racX*, genes whose predicted products exhibit sequence similarity to known racemases. Strains mutant for *ylmE* or *racX* alone showed a modest delay in pellicle disassembly (Fig. S4). However, pellicles formed by cells doubly mutant for the putative racemases were significantly delayed in disassembly (Fig. S4). Conversely, cells engineered to overexpress *ylmE* were blocked in biofilm formation (Fig. S5). Conditioned medium from the double mutant was also ineffective in inhibiting biofilm formation, in contrast to conditioned medium from the wild type (Fig. 2B). Next, we asked whether D-amino acids are produced in sufficient abundance to account for disassembly of mature biofilms. Accordingly, we carried out liquid chromatography-mass spectrometry followed by the identification of the D-amino acids using derivatization with Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (L-FDAA) on conditioned medium collected at early and late times after pellicle formation. D-tyrosine (6 µM), D-leucine (23 µM), and D-methionine (5 µM) were present at concentrations at or above those needed to inhibit biofilm formation by day 6 but at concentrations of <10 nM at day 3. In contrast, the *ylmE racX* double mutant was blocked in D-tyrosine production and impaired in D-leucine production at day 6 (Table S1).

How do D-amino acids disassemble biofilms? D-amino acids did not inhibit growth (Fig. S6), nor did they inhibit the expression of the matrix operons *epsA-O* and *yqxM-sipW-tasA* (Fig. S7). D-amino acids are incorporated into the peptide side chains of peptidoglycan in place of the terminal D-alanine (9). Using ^{14}C -D-tyrosine, we confirmed that the D-tyrosine (but not ^{14}C -L-proline) was incorporated into the cell wall (Fig. S8), with incorporation commencing at day 3 (Fig. S9). Finally, and in keeping with the idea D-amino acids act via their incorporation into the wall, the effects of D-tyrosine and the D-amino acid mixture were prevented by D-alanine (Fig. 1K–L).

We hypothesized that TasA fibers are anchored to the cell wall and that the incorporation of biofilm-disassembling D-amino acids into the cell wall might disengage the fibers from their anchor. To investigate this possibility, we examined the localization of a functional fusion of TasA with the fluorescent reporter mCherry. Treatment with D-tyrosine had little effect on the accumulation of TasA-mCherry (Fig. S10). In contrast, when the cells were washed by centrifugation, resuspended and then examined by fluorescence microscopy, untreated cells, which were often in clumps, were intensely decorated with TasA-mCherry (Fig. 3A). In contrast, D-tyrosine-treated cells, which were largely unclumped, showed only low levels of fluorescence (17-fold lower; Table S2). Similar results were obtained with D-leucine and

with the D-amino acid mixture. We also carried out electron microscopy with gold-labeled anti-TasA antibodies to visualize unmodified TasA. TasA fibers were anchored to the cells of untreated pellicles (Fig. 3B, images 1 and 2). In contrast, cells treated with D-tyrosine consisted of a mixture of cells that were largely undecorated with TasA fibers and free TasA fibers or aggregates of fibers that were not anchored to cells (Fig. 3B, images 3–6).

Next, we isolated D-amino acid resistant mutants (Fig. 4A). Wrinkled papillae appeared spontaneously on the flat colonies formed during growth on solid medium containing D-tyrosine (Fig. 4A) or D-leucine (Fig S2). When purified, these spontaneous mutants gave rise to wrinkled colonies and pellicles in the presence of individual D-amino acids. We isolated several such mutants and found that they contained mutations in the 3' region of *yqxM*. Two mutations that conferred resistance to D-tyrosine were examined in detail. *yqxM2* was an insertion of G:C at base pair 728 and *yqxM6* was a deletion of A:T at base pair 569 (Fig. 4B). The presence of *yqxM2* and *yqxM6* restored clumping and cell decoration by TasA-mCherry to cells treated with D-tryosine (Fig. 3D, Fig. S12, see above). Because YqxM is required for the association of TasA with cells (6), the discovery that the biofilm-inhibiting effect of D-amino acids could be overcome by mutants of YqxM reinforces the view that the effect of D-amino acid incorporation into the cell wall is to impair the anchoring of the TasA fibers to the cell. A domain near the C-terminus of YqxM could trigger the release of TasA in response to the presence of D-amino acids in the cell wall.

Finally, we wondered whether D-amino acids would inhibit biofilm formation by other bacteria. The pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* form biofilms on plastic surfaces (11), which can be detected by washing away unbound cells and staining the bound cells with Crystal Violet. D-tyrosine and the D-amino acid mixture were effective in preventing biofilm formation (Fig. S13), whereas L-tyrosine and L-amino acids had no effect. Furthermore, the effect of D-amino acids was prevented by the presence of D-alanine (Fig. S13), suggesting that D-amino acids acted to block biofilm formation by replacement of D-alanine in the peptide side chain. Given that many bacteria produce D-amino acids, D-amino acids may provide a general strategy for biofilm disassembly. If so, then D-amino acids might prove widely useful in medical and industrial applications for the prevention or eradication of biofilms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

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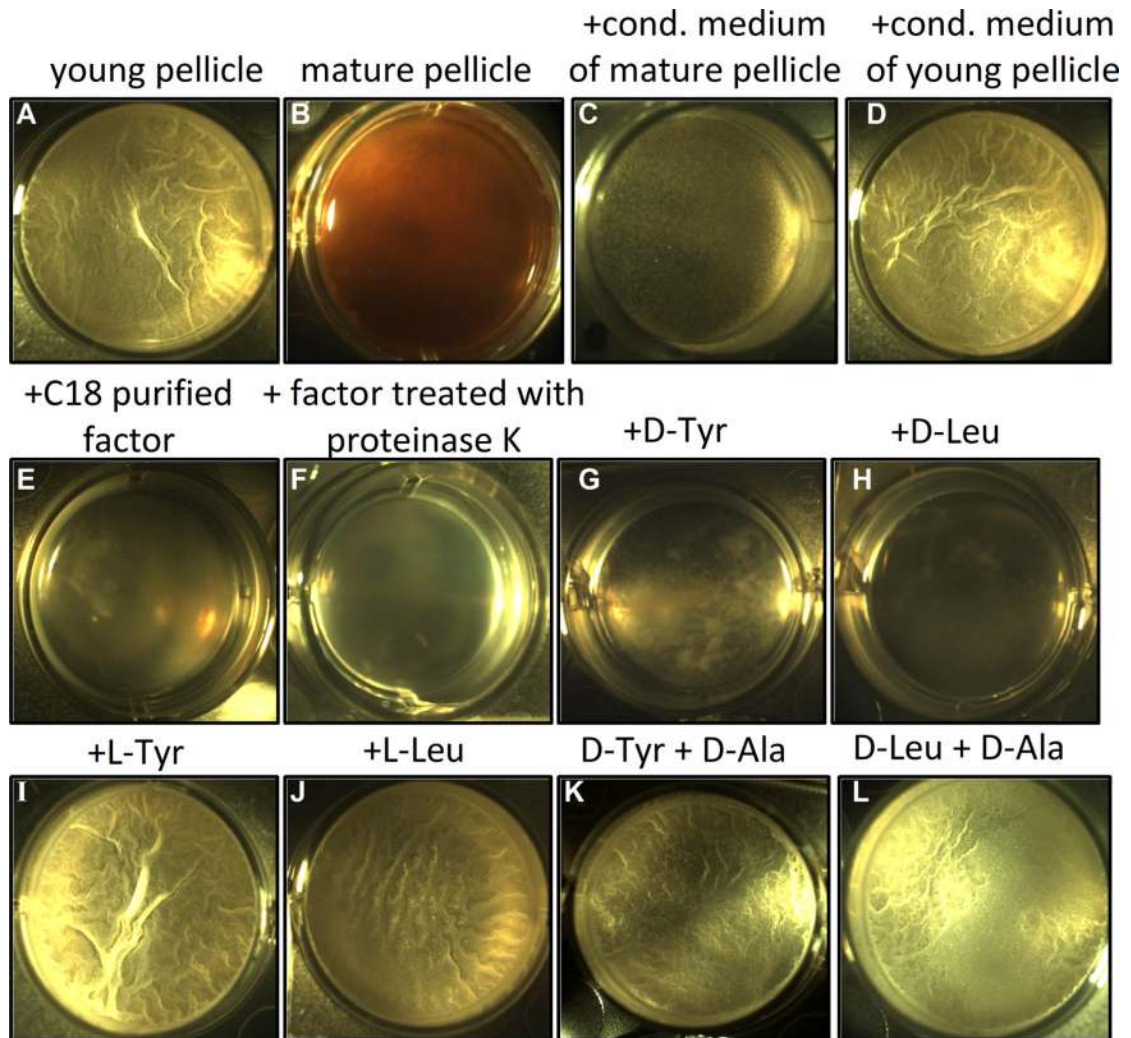
Figure 1. Conditioned medium blocks pellicle formation

B. subtilis strain NCIB3610 that were grown at 22 °C in 12-well plates in liquid biofilm-inducing medium for 3 days (A) or for 8 days (B). Panels C and D show cells grown for 3 days in medium to which had been added a dried and resuspended methanol eluate (1:100 v/v) from a C18 Sep Pak column that had been loaded with conditioned medium from a 6–8 day-old culture (C) or a 3 day-old culture (D). The final concentration of concentrated factor added to the wells represented a 1:4 dilution on a volume basis of the original conditioned medium. In Panel E the factor was further purified on the C-18 column by step-wise elution with methanol. Shown is the result of adding 3 µl of the 40% methanol eluate. In Panel F prior to addition to fresh medium the 40% methanol eluate was incubated with Proteinase K beads. In panel G-L: wells containing MSgg medium supplemented with D-tyrosine (3 µM), D-leucine (8.5 mM), L-tyrosine (7 mM), L-leucine (8.5 mM), D-tyrosine (3 µM) and D-alanine (10 mM), or D-leucine (8.5 mM) and D-alanine (10 mM) were inoculated with strain NCIB3610 and incubated for 3 days. In all panels, the diameter of the wells was 22.1 mm.
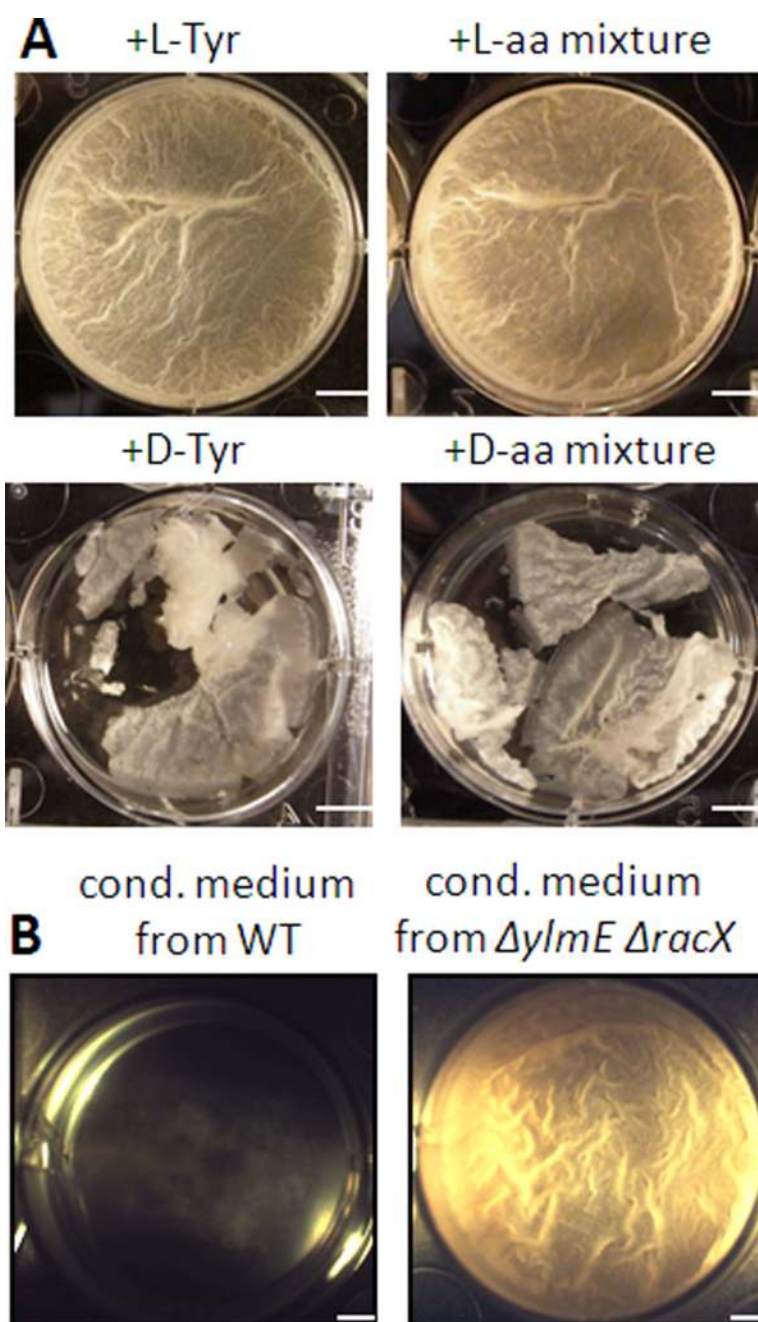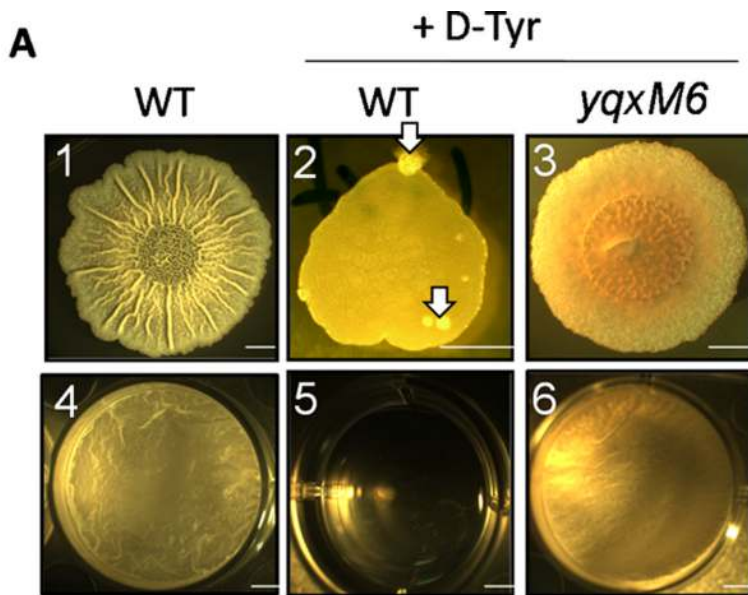
Figure 2. D-amino acids mimic the effect of conditioned medium
Panel A shows 3 day-old cultures to which had been added (as a 10 µl drop to the surface of the pellicles to achieve the indicated final concentration) L-tyrosine (7 mM), a mixture of L-amino acids (5 mM each), D-tyrosine (3 µM) or a mixture of D-amino acids (2.5 nM each). Panel B shows the effect of concentrated Sep Pak C-18 column eluate from conditioned medium from an 8-day-old culture from the wild type or from a strain (IKG55) doubly mutant for *ylmE* and *racX*. Scale bar is 7 mm.
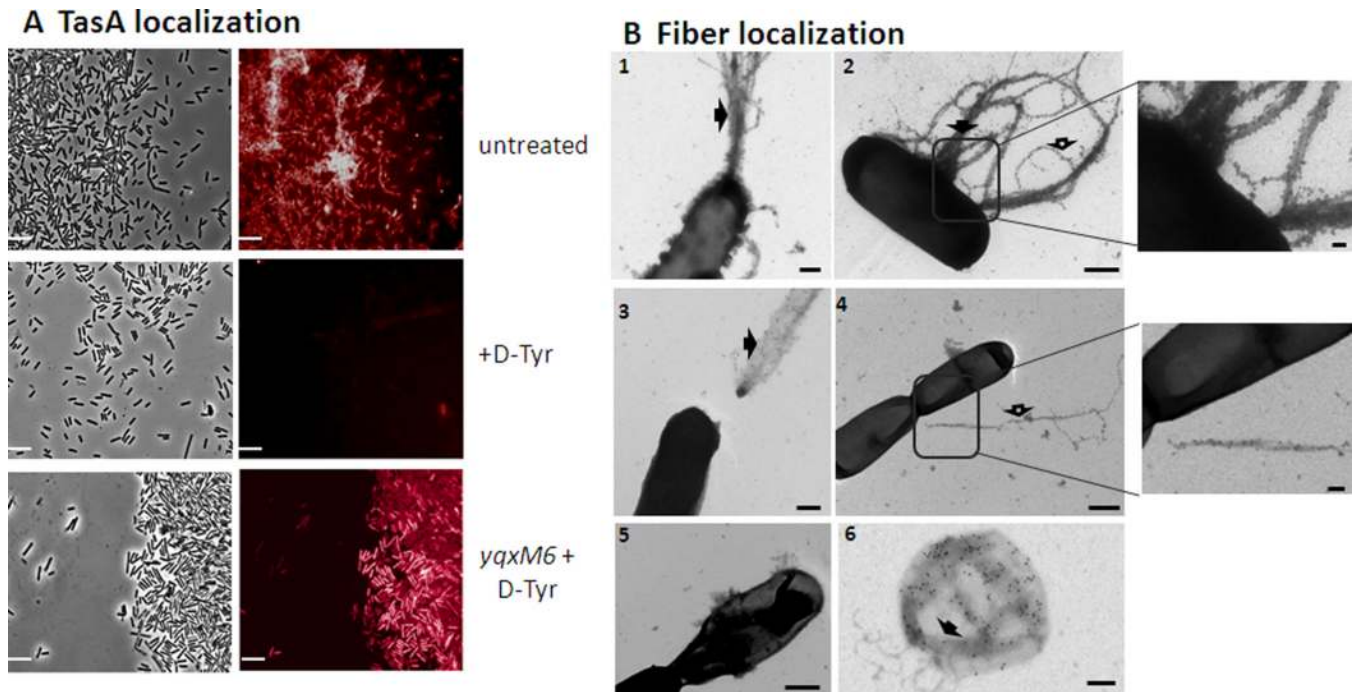
Figure 3. D-tyrosine causes the release of TasA fibers
Panel A shows cell association of TasA-mCherry by fluorescence microscopy. Wild-type
cells and *yqxM6* (IKG51) mutant cells containing the *tasA-mCherry* fusion were grown to
stationary phase (OD=1.5) with shaking in biofilm-inducing medium in the presence or
absence (untreated) of D-tyrosine (6 µM) as indicated, washed in PBS, and visualized by
fluorescence microscopy. Scale bar is 4 µm. Panel B shows cell association of TasA fibers
by electron microscopy. 24-hour-old cultures were incubated without (images 1 and 2) or
with (images 3–6) D-tyrosine (0.1 mM) for an additional 12 hours. TasA fibers were stained
by immunogold labeling using anti-TasA antibodies, and visualized by transmission electron
microscopy. Filled arrows indicate fiber bundles; open arrows indicate individual fibers. The
scale bar is 500 nm. The scale bar in the enlargements of images 2, 4 and in image 6 is 100
nm. Images 1 and 2 show fiber bundles attached to cells, images 3, 4 and 6 show individual
fibers and bundles detached from cells, and images 3–5 show cells with little or no fiber
material. For further details see SOM.

Figure 4. Biofilm formation by YqxM mutants resistant to D-tyrosine

Panel A shows wild type or *yqxM6* (IKG51) mutant cells grown on solid (1–3) or liquid (4–6) biofilm-inducing medium. Cells were grown in the absence (1, 4) or presence (2, 3, 5, 6) of D-tyrosine (3 µM). Arrowheads in 2 indicate papillae of spontaneous resistances mutants. Scale bar is 3 mm. Panel B shows the C-terminal amino acid sequences for wild type YqxM and the indicated frame-shift mutants.