

# Osmotic pressure can regulate matrix gene expression in *Bacillus subtilis*

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## Summary

Many bacteria organize themselves into structurally complex communities known as biofilms in which the cells are held together by an extracellular matrix. In general, the amount of extracellular matrix is related to the robustness of the biofilm. Yet, the specific signals that regulate the synthesis of matrix remain poorly understood. Here we show that the matrix itself can be a cue that regulates the expression of the genes involved in matrix synthesis in *Bacillus subtilis*. The presence of the exopolysaccharide component of the matrix causes an increase in osmotic pressure that leads to an inhibition of matrix gene expression. We further show that non-specific changes in osmotic pressure also inhibit matrix gene expression and do so by activating the histidine kinase KinD. KinD, in turn, directs the phosphorylation of the master regulatory protein Spo0A, which at high levels represses matrix gene expression. Sensing a physical cue such as osmotic pressure, in addition to chemical cues, could be a strategy to non-specifically co-ordinate the behaviour of cells in communities composed of many different species.

## Introduction

The ability to form structured communities of cells (biofilms) is a widespread feature of bacteria (O'Toole *et al.*, 2000; Branda *et al.*, 2005; Kolter and Greenberg, 2006). Cells in biofilms are held together by an extracellular matrix

that consists of polysaccharide and protein and sometimes DNA (Branda *et al.*, 2005; Lopez *et al.*, 2010) but whose composition can vary greatly depending on the conditions and the species present (O'Toole *et al.*, 2000; Branda *et al.*, 2005). Given the chemical diversity of the matrix, how might a mixed community regulate the amount of matrix made? We reasoned that, in addition to chemical cues, the physical properties of diverse matrices might provide a common cue for regulating matrix synthesis. In particular, we focused on osmotic pressure because the high concentration of exopolysaccharide (EPS) polymers (up to 90% of a biofilm's dry mass (Flemming and Wingerder, 2010) would be expected to increase the osmotic pressure and there is ample precedent that osmotic pressure can regulate gene expression (Russo and Silhavy, 1991; Ruzal and Sanchez-Rivas, 1998).

An attractive organism in which to study biofilm development is the spore-forming bacterium *Bacillus subtilis*. *B. subtilis* forms highly structured colonies on semi-solid surfaces and thick pellicles at the air–liquid interface of standing cultures as a consequence of extracellular matrix production (Branda *et al.*, 2001; Lemon *et al.*, 2008). The main components of the *B. subtilis* extracellular matrix are EPS, which is synthesized by enzymes encoded by the *epsA-O* operon and amyloid-like fibres, which are encoded by *tapA-sipW-tasA* (Branda *et al.*, 2004; 2006; Romero *et al.*, 2010). Both operons are subject to negative regulation by the repressor SinR (Chu *et al.*, 2006). Derepression of the matrix operons is brought about by the antirepressor SinI (Bai *et al.*, 1993; Chai *et al.*, 2008), whose synthesis is turned on by the phosphorylated form of the regulatory protein Spo0A, which also directs the transcription of genes that govern entry into sporulation (Fujita *et al.*, 2005; Fujita and Losick, 2005). At least four histidine kinases contribute to the phosphorylation of Spo0A via a multicomponent phosphorelay (Burbulys *et al.*, 1991; Jiang *et al.*, 2000). Low levels of Spo0A~P turn on SinI synthesis but are insufficient to activate genes contributing to sporulation, which have weak binding sites for the phosphoprotein (Fujita *et al.*, 2005). High levels of Spo0A~P, on the other hand, repress the gene for SinI while turning on sporulation gene expression (Chai *et al.*, 2011).

In earlier work, an intriguing connection was observed between EPS production and the onset of spore formation

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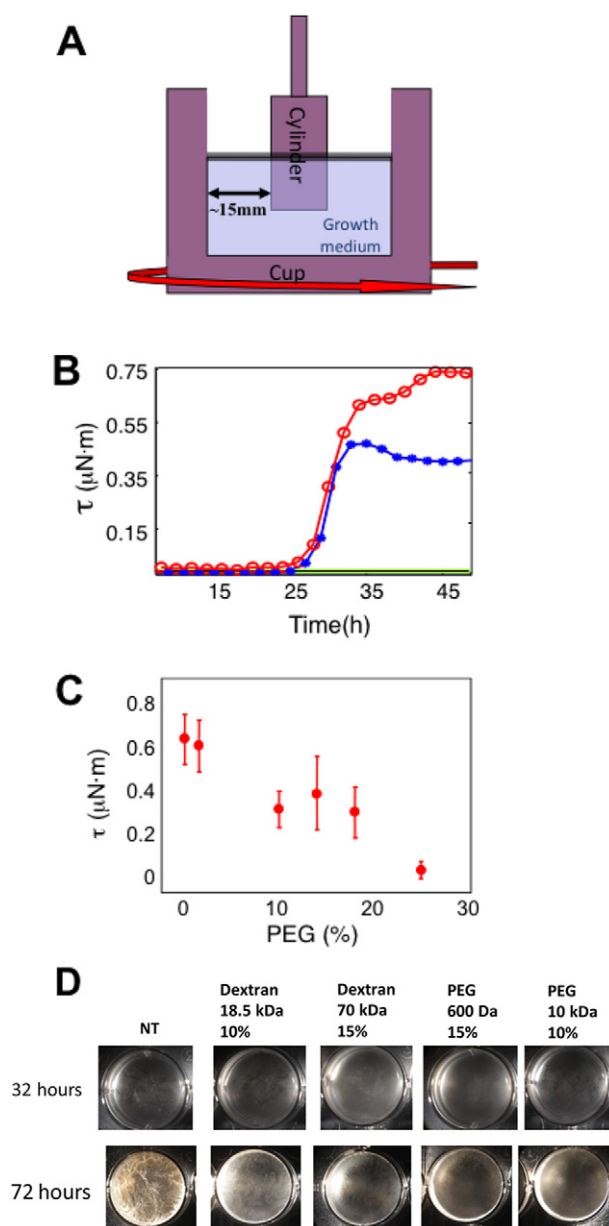
during biofilm formation. Efficient entry into sporulation was found to depend on matrix production in a manner that was bypassed by a mutation in the gene for the histidine kinase KinD (Aguilar *et al.*, 2010). Thus, KinD was inferred to be a checkpoint protein that linked sporulation to EPS production. Some histidine kinases are known to be bifunctional in that they exhibit competing phosphatase and kinase activities (Zhu *et al.*, 2000; Kostakioti *et al.*, 2009). It was proposed that KinD is such a bifunctional kinase and that at low EPS levels, KinD is principally a phosphatase that keeps Spo0A~P concentrations low and that at high EPS levels it becomes a kinase, allowing the phosphoprotein to accumulate to high concentrations (Aguilar *et al.*, 2010).

But how EPS production effects such a switch was mysterious. Here we report that a non-specific physical cue, increasing osmotic pressure from the accumulation of EPS during biofilm formation, stimulates the kinase activity of KinD, causing Spo0A~P levels to rise. This rise represses the gene for SinI and hence matrix gene expression and instead turns on genes for entry into sporulation. Thus, a physical property of the matrix, osmotic pressure, co-ordinates the behaviour of cells in the biofilm.

## Results and discussion

### Polymer addition inhibits biofilm robustness and matrix gene expression

As *B. subtilis* biofilms develop, the amount of wrinkling and overall thickness increase and there is an apparent concomitant increase in robustness. We have developed a quantitative physical assay for biofilm robustness that involves measuring a pellicle's shear strength as it grows in small circular wells. Pellicles were grown at 30°C in a cylindrical cell on an Ares G2 strain-controlled rheometer (Fig. 1A). A cylindrical bob coupled to a torque sensor was placed at the centre of the well. Tests of pellicle strength were conducted by periodically (0.1 Hz) rotating the well around its symmetrical axis at small angles ( $\leq 0.6^\circ$ ) and measuring the torque on the cylinder as shown in Fig. 1A. The maximal torque measured per period is defined as the oscillatory torque. Under these experimental conditions, the oscillatory torque reflects the viscoelastic properties of the biofilm. Thus, measuring the oscillatory torque as pellicles form can be used as an assay to probe the strength and hence the robustness of biofilms. Under our conditions, the first measurable increase in robustness occurred about 26 h after inoculation (red curve in Fig. 1C). After that, the strength increased steadily for ~8 h before levelling off. We wished to ask how a change in the physical environment influenced biofilm development. Therefore, we performed torque measurements as a pellicle was forming in the presence of 20% (by mass) of



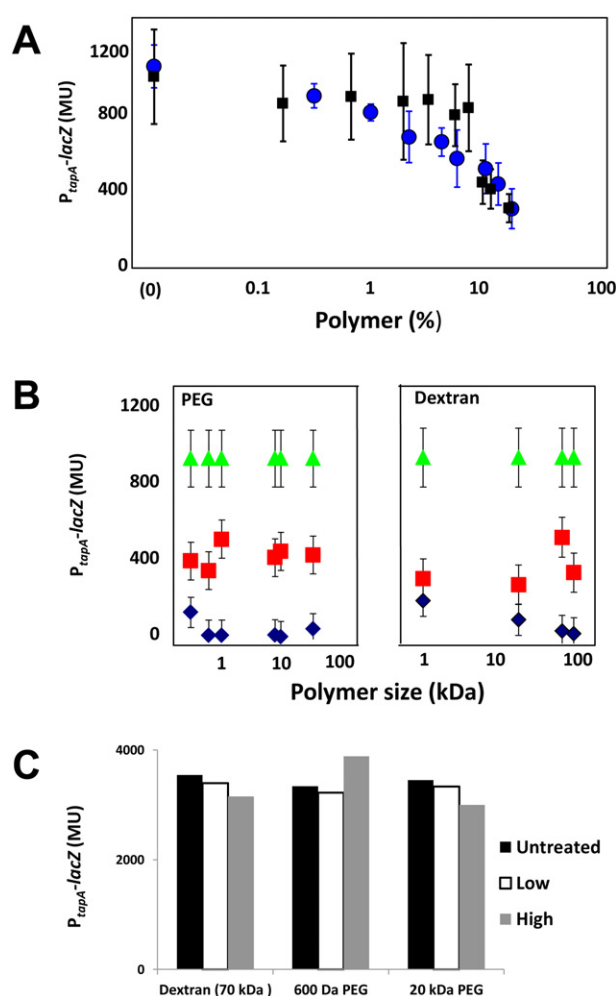
**Fig. 1.** Real-time measurements of pellicle strength. A. Pellicles were grown inside a cuvette cell on top of an Ares G2 strain controlled rheometer. The diameter of the outside well was 34 mm and that of the inside cylinder was 15 mm. The set-up was held at a constant temperature of 30°C. B. Oscillatory torque,  $\tau$ , was measured at a strain of 0.5% approximately every 2 h for wild-type bacteria in MSgg (red), MSgg supplemented with 20% by mass of 20 kDa PEG (blue), MSgg with 15% by mass of PEG with no bacteria (green) and pure MSgg inoculated with a matrix mutant that was not capable of synthesizing exopolysaccharide (black). The pellicle began to show rigid behaviour after ~30 h for both pure and PEG supplemented medium. Note, however, that the supplemented medium saturated faster and did not reach the level of robustness of the pure medium. C. Oscillatory torque,  $\tau$ , for wild-type pellicles grown in MSgg medium supplemented with 20 kDa PEG at 0–25% mass fractions. D. 3610 was grown in a liquid biofilm medium for 3 days with/without polymer addition. Pictures were taken using a SPOT camera (Diagnostic Instruments, USA). This figure is available in colour online at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).

20 kDa polyethylene glycol (PEG). Surprisingly, while the initial stages of pellicle formation did not change, there was a marked change at later times; the strengthening stopped earlier and the mature pellicle was ~40% weaker (blue curve in Fig. 1B). In fact, the pellicles weakened monotonically with the amount of PEG added to the medium (Fig. 1C). [A similar correlation between polymer concentration and pellicle weakening was obtained using the maximal torque (yield torque) a pellicle can withstand before it ruptures (data not shown).] Moreover, pellicles grown in the presence of PEG had a visible defect in robustness (Fig. 1D). As shown Fig. 1D even though rates of strengthening during the initial stages were similar, biofilms grown in the presence of PEG stopped strengthening earlier than those grown in medium without PEG.

Why did the addition of PEG impair the development of biofilm robustness? Since biofilm strength is associated with matrix, one possibility was that the addition of PEG led to a reduction in matrix gene expression and hence the production of matrix. To investigate this we asked whether the addition of PEG would inhibit the expression of the matrix operon *tapA-sipW-tasA* (henceforth simply *tapA*). Cells were grown in biofilm-inducing medium (MSgg) without shaking to facilitate pellicle formation. The medium was modified by addition of PEG or Dextran over a range of concentrations. After 48 h we harvested the cells and measured the expression of *tapA*. The results show that increasing the concentration of polymer caused a marked reduction in matrix gene expression (Fig. 2A) and pellicle robustness (Fig. 1D), while having little or no effect on cell growth (Fig. S1).

#### The effect of polymer correlates with changes in osmotic pressure

To investigate the basis for the polymer-induced decrease in the expression of *tapA*, we took advantage of the unique properties of polymeric solutions to differentiate between the effects of multiple physical parameters. To do this, we tested the effects of both PEG and dextran over a range of molecular sizes and concentrations as well as various concentrations of the dextran subunit dextrose. This enabled us to distinguish among the contributions of viscosity, osmotic pressure, diffusivity, ionic strength and chemical composition. Thus, for instance, solutions of 2.5% PEG of molecular weight 0.3 kDa and 15% PEG of molecular weight 20 kDa exert similar osmotic pressures but their viscosities differ by more than one order of magnitude. Given that we could reproduce the effect for both small (dextrose or PEG 600 Da) and large (Dextran 110 kDa and PEG 35 kDa) molecules, we concluded that there was no correlation with size and diffusivity. The diffusivity of small molecules is almost completely unaltered by the addition of polymers and therefore cannot

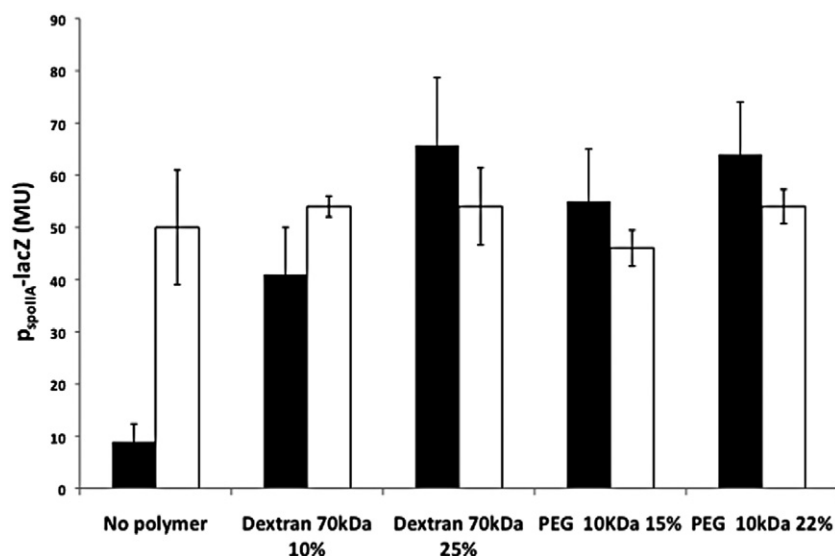


**Fig. 2.** Osmotic pressure regulates matrix gene expression.

A.  $P_{tapA}$  activity in a wild-type strain carrying  $P_{tapA-lacZ}$  (RL4582) was measured as a function of the mass fraction of 20 kDa PEG (blue) and 70 kDa dextran (black) added to the medium. B.  $P_{tapA}$  activity in a wild-type strain carrying  $P_{tapA-lacZ}$  (RL4582) was measured in medium supplemented with various concentrations of different-sized PEG (right) and Dextran (left) polymers prepared at set osmotic pressures: dark blue diamonds, magenta squares and green triangles corresponded respectively to 5–8, 1–3 and 0 atm additional osmotic pressure exerted by the polymers. C.  $P_{tapA-lacZ}$  activity was measured in a *sinR* mutant (in which *tapA* is expressed constitutively) (RL4585). The curves represent three experiments, performed in duplicates, in which different polymers were used to increase the osmotic pressure; 70 kDa Dextran 600 Da PEG and 20 kDa PEG.

Error bars are the standard deviation. This figure is available in colour online at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).

explain the effect we observed. In contrast, the diffusivity of large molecules depends strongly on the polymer size. As both PEG and dextran caused similar effects we also concluded that chemical composition of the polymeric additives was much less significant than the osmotic pressure (Fig. S2). Additionally, the observed effects cannot be due to changes in ionic strength because PEG is neutral. We also ruled out any systematic dependence on



**Fig. 3.** Polymer stimulates sporulation gene expression in a KinD-dependent manner. A wild-type strain (IKG10; black columns) and its *kinD* mutant derivative (IKG603; white columns), both carrying a sporulation gene reporter  $P_{spolIA}$ -*lacZ*, were grown with the indicated polymers. The columns represent three experiments, performed in duplicates. Error bars are the standard deviation.

viscosity of the polymeric solutions (Fig. S3). Lastly and importantly, we found that the level of matrix gene expression correlated strongly with the osmotic pressure exerted by the polymers (Figs 2B and S2).

The *tapA* operon is under the negative control of the repressor SinR (Kearns *et al.*, 2005; Chai *et al.*, 2008). If osmotic pressure inhibits expression (blocks derepression) of the operon, then it should exert little or no effect in a *sinR* mutant in which *tapA* is expressed constitutively. As evidence that this is the case, pellicle morphology in a *sinR* mutant was largely unchanged in the presence of polymers (Fig. S4). Also, *tapA* transcription was insensitive to osmotic pressure in a mutant lacking the repressor (Fig. 2C) (as compared with the wild type in Fig. 2C). (Note that the level of *tapA* expression is much higher in a *sinR* mutant than in the wild type as seen from a comparison of the scales between panels B and C.) *In toto*, the results so far indicate that osmotic pressure acts at or upstream of SinR in the pathway governing *tapA* transcription.

Next, we asked whether in wild-type cells increasing osmotic pressure would stimulate sporulation gene expression, which requires high levels of Spo0A~P (Fujita and Losick, 2005; Fujita *et al.*, 2005). Transcription of a sporulation-specific promoter (*spolIA*) significantly increased following the addition of various polymers (Fig. 3). Moreover, cells grown in biofilm-inducing medium supplemented with various polymers sporulated prematurely (Fig. S5).

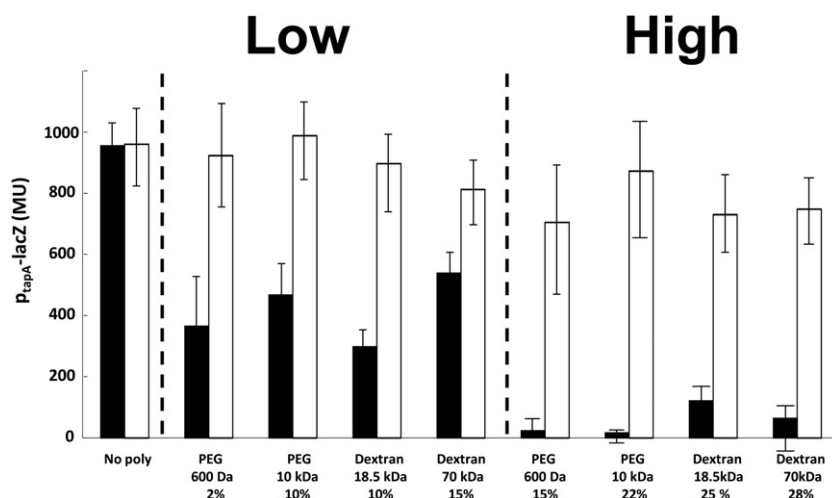
#### The effect of polymer is dependent on KinD

Previous work has indicated the histidine kinase KinD is a checkpoint protein that links the levels of Spo0A~P to the accumulation of extracellular matrix (Aguilar *et al.*, 2010).

As a result, KinD mutant cells exhibit a subtle biofilm defect, and sporulate prematurely (Vlamakis *et al.*, 2008). It was proposed that KinD is principally a phosphatase at low levels of matrix, keeping Spo0A~P at low to moderate concentrations, and principally a kinase at high matrix levels, causing Spo0A~P to reach high concentrations. At low to moderate Spo0A~P concentrations, matrix genes would be ON and sporulation genes OFF whereas at high matrix levels, matrix genes would be OFF (via repression of the gene for SinI) and genes governing entry into sporulation would be ON (Chai *et al.*, 2011). We therefore wondered whether the presence of the extracellular matrix is sensed by KinD as polymer-induced osmotic pressure. In contrast to the wild type, increasing osmotic pressure in a KinD mutant failed to inhibit expression of the matrix gene *tapA* (Fig. 4). In addition, a KinD mutant was largely unaltered in the pellicle morphology with or without added polymers (Fig. S6). Also, transcription from the sporulation promoter *spolIA* was higher in a KinD mutant than in the wild type in the absence of added polymer (in keeping with the idea that KinD is principally a phosphatase that keeps Spo0A~P levels low when unstimulated) (Fig. 3). This high level of sporulation transcription seen in the absence of KinD was not further increased by raising the osmotic pressure (Fig. 3). The effect was specific to KinD as mutants lacking KinA, KinB or KinC were unaltered in their response to changes in osmotic pressure (Fig. 5). This suggests that KinD is an osmosensor whose kinase activity increases and whose phosphatase activity decreases in response to increasing osmotic pressure, raising Spo0A~P levels.

A distinctive feature of KinD is that its activity depends on a lipoprotein called Med (Banse *et al.*, 2011). We therefore wondered whether KinD activity would be dependent on Med in our osmosensing assay. We confirmed that this





**Fig. 4.** Polymer inhibits matrix gene expression in a KinD-dependent manner. A wild-type strain (RL4582; black columns) and its *kinD* mutant derivative (IKG602; white columns), both carrying a matrix gene reporter  $P_{tapA}$ -lacZ were grown with the indicated polymers.  $P_{tapA}$  activity was measured under conditions of low (Low) and high (High) osmotic pressure. The columns represent three experiments, performed in duplicates, in which different polymers described in Fig. 2B were used to increase the osmotic pressure. Error bars are the standard deviation.

was indeed the case in experiments similar to those of Fig. 3 (data not shown).

If increasing osmotic pressure was elevating Spo0A~P levels, then in a rich medium (LB) in which the levels of the phosphoprotein are normally very low, polymer addition should turn on genes whose transcription requires low to moderate levels of Spo0A~P and are normally not expressed in LB medium. As shown in Fig. 6, the addition of either PEG or Dextran to LB medium significantly increased the transcription of *sdp*, an operon that is under the direct control of Spo0A~P and activated at low levels of the phosphoprotein (Gonzalez-Pastor *et al.*, 2003; Fujita *et al.*, 2005). This induction was dependent on KinD but not on KinA, KinB or KinC (Fig. 6). In sum, our results are consistent with a model in which KinD is an osmosen-

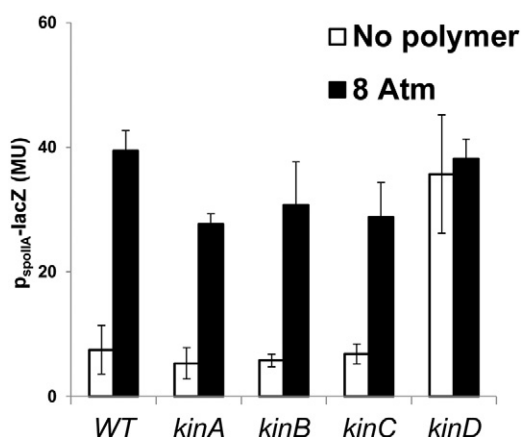
sor that detects increases in osmotic pressure from the synthesis of matrix and in response stimulates the phosphorylation of Spo0A.

Finally, we investigated the idea that during biofilm formation KinD is responding to increasing osmotic pressure from the accumulation of matrix polymers. That is, whether the physical property of the matrix is an environmental signal that is detected by cells in the community and used to co-ordinate their behaviour. If polymer-induced increases in osmotic pressure are indeed regulating matrix production and the onset of sporulation, then in biofilm-inducing medium a mutant blocked in EPS production ( $\Delta eps$ ) (Branda *et al.*, 2004; Chu *et al.*, 2006) should have low levels of Spo0A~P. Indeed, as seen previously (Vlamakis *et al.*, 2008), in the absence of EPS, matrix gene transcription was elevated (Fig. 7A) [recall that high levels of Spo0A~P inhibit matrix gene transcription (Chai *et al.*, 2011)] and sporulation transcription was markedly decreased (Fig. 7B) (Aguilar *et al.*, 2010). These effects were reversed by artificially raising the osmotic pressure to 2 atm and 5–8 atm (based on using several different polymers; Fig. 7A and B) in a manner that was dependent on KinD (Fig. 7C and D). In sum, these findings reinforce the view that KinD is an osmosensor whose kinase activity is stimulated and whose phosphatase activity decreased by the increase in osmotic pressure caused by EPS synthesis.

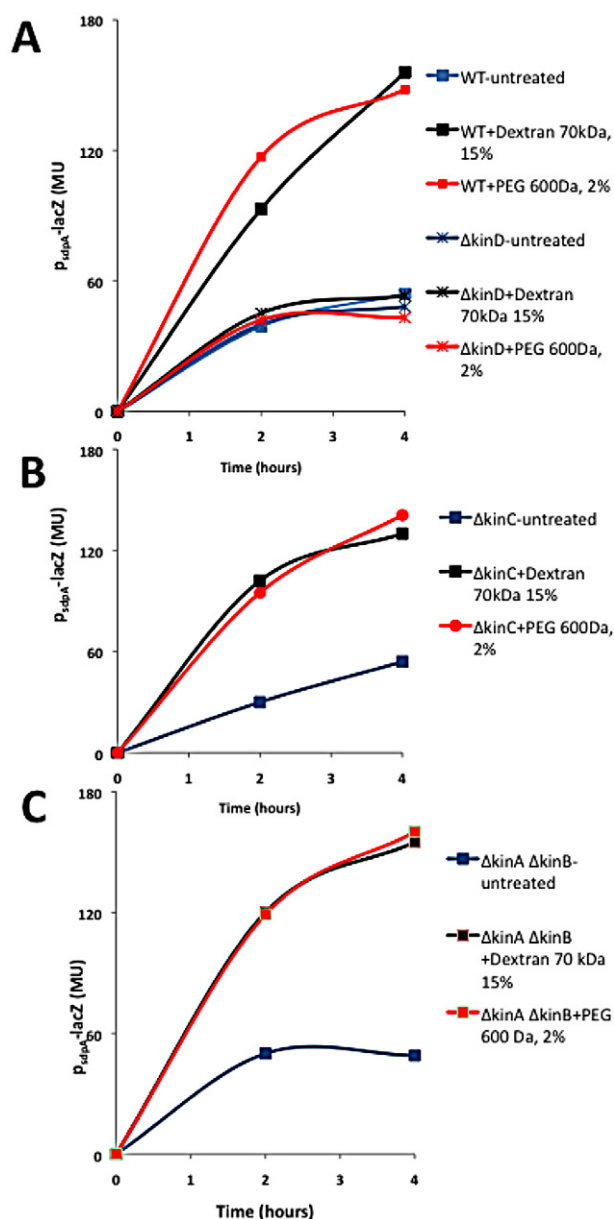
As a final test of the idea that KinD activity is controlled by osmotic pressure, we measured the osmotic pressure within the pellicle itself. Using a freezing-point osmometer (AI model 3300), we found that the osmotic pressure of the pellicle was higher than that of the medium by  $3 \pm 1$  atm.

#### Evidence that osmosensing depends on a transmembrane segment

Interestingly, EnvZ of *Escherichia coli*, a histidine kinase that also senses and responds to changes in osmotic



**Fig. 5.** The uncoupling of sporulation gene expression from increasing osmotic pressure is specifically dependent on KinD. Strain 3610 and its kinase mutant derivatives, *kinA* (IKG604), *kinB* (IKG605), *kinC* (IKG606), *kinD* (IKG603), all carrying  $P_{spoIIA}$ -lacZ (a sporulation promoter), were grown in biofilm-inducing medium.  $P_{spoIIA}$  activity is expressed in Miller units. The columns represent three experiments, performed in duplicates, in which different polymers described in Fig. 2B were used to increase the osmotic pressure. Error bars are the standard deviation.



**Fig. 6.** Stimulatory effect of osmotic pressure on Spo0A-P-directed gene expression is specifically dependent on KinD. Strain 3610 carrying  $P_{sdpA}$ - $lacZ$  (IKG609) and its derivatives with the kinase mutations  $\Delta kinC$  (IKG611),  $\Delta kinA \Delta kinB$  (IKG612) and  $kinD$  (IKG610) were grown in LB (non-sporulation) medium in a shaking culture to early stationary phase.  $P_{sdpA}$  activity is expressed in Miller units. The curves represent three experiments, performed in triplicates. Error bars are the standard deviation.

pressure, is known to function as both a kinase and a phosphatase (Russo and Silhavy, 1991; Hsing *et al.*, 1998). Earlier work had shown that the transmembrane segments of EnvZ play an important role in this osmosensing (Tokishita *et al.*, 1992). Intriguingly, we note a 16-residue long region of similarity between transmembrane segments of the proteins: IVtL-LFasLVttYL in EnvZ versus IVILnILFi-LVlyYL in KinD (upper case letters

indicate residues that are identical between the two sequences; the segment of similarity is highlighted in yellow in Fig. 8A).

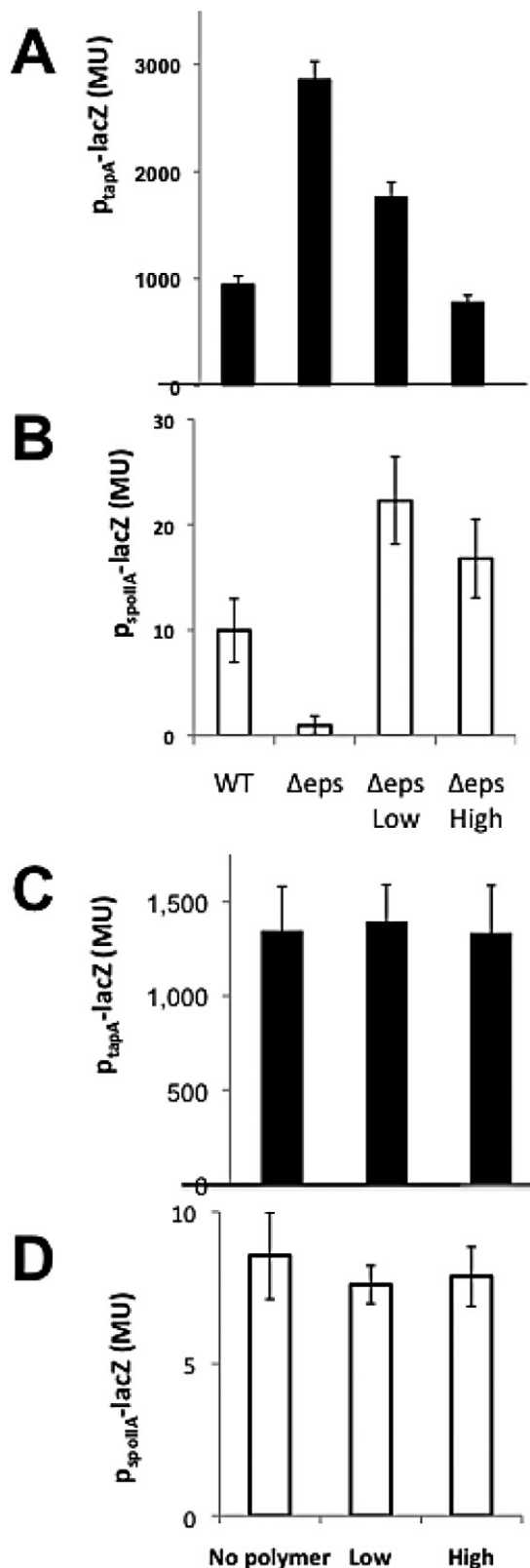
We created alanine substitutions for several of the residues in the transmembrane segment, asking whether the mutants would be impaired in osmosensing. As shown in Fig. 5, replacing two highly conserved leucine residues with alanine (L260A and in L264A) resulted in the partial loss of KinD's ability to turn matrix genes OFF (Fig. 8B) and sporulation promoters OFF (Fig. 8C). Furthermore, a double mutant for L260 and L264 was conspicuously insensitive to osmotic pressure as judged by colony morphology (Fig. S6) and transcription of *tapA* and *spoIIA* (Fig. S7).

Substitution of the entire transmembrane segment with a transmembrane segment of KinB, a transmembrane kinase that is not required for osmosensing (Fig. 8B and C), resulted in complete loss of osmosensing by KinD.

Interestingly, KinD has a CACHE domain that appears to respond to a small molecule released by plant roots and to glycerol in high concentration (Chen *et al.*, 2012; M. Shemesh and R. Losick, unpubl. results). KinD mutant for the CACHE domain was largely unimpaired in osmosensing (Fig. 8B and C). Conversely, however, the L260A and L264A mutants of transmembrane domain II were not blocked in responding to glycerol (data not shown). That these mutants remained active in responding to glycerol in a CACHE domain-dependent manner indicates that the L260A and L264A substitutions had not completely inactivated KinD. Rather, the effect of the substitutions was specific to osmosensing. We therefore hypothesize that KinD is a bifunctional kinase that can independently sense a small molecule(s) via its CACHE domain and osmotic pressure via its transmembrane segment.

Our results suggest that *B. subtilis* uses the polymers it produces during biofilm formation as a physical cue to co-ordinate the behaviour of cells in the community. Conceivably, the physical properties of matrix polymers are also exploited for synchronizing development in biofilms composed of multiple species. As an indication of the plausibility of this idea, EPS extracted from *Pseudomonas aeruginosa* and *E. coli* pellicles was observed to substitute for *B. subtilis* EPS in inhibiting matrix gene expression in an *eps* mutant of *B. subtilis* (Fig. 9). It will be interesting to see whether the physical properties of biofilms are used as a developmental cue by diverse kinds of biofilm-forming bacteria, including those that form communities in consortia with other bacteria.

Finally, we comment on the approach we devised to measure the rheological properties of the biofilm. Probing physical properties ordinarily requires applying physical forces: to measure the strength of a material, it needs to be stretched or squeezed. Thus, biofilm rheological assays usually involve either measuring local microscopic



**Fig. 7.** Increasing osmotic pressure mimics the effect of matrix production on matrix and sporulation gene expression in a matrix mutant.

A and B. The activities of  $P_{tapA}$ -lacZ in a wild-type strain (RL4582) and its  $\Delta eps$  (IKG600) mutant derivative (A) and of  $P_{spoIIA}$ -lacZ in a wild-type strain (IKG10) and its  $\Delta eps$  derivative (IKG601) (B) were determined without added polymer and with osmolarity increased (using the polymers described in Fig. 2B) to 2 atm (Low) and 5–8 atm (High). The results shown are the average of using multiple polymers in triplicate.

C and D. The activities of  $P_{tapA}$ -lacZ in a  $\Delta eps$  mutant lacking *kinD* (IKG607) (C) and of  $P_{spoIIA}$ -lacZ in an  $\Delta eps$  mutant lacking *kinD* (IKG608) (D) were determined without added polymer and at 2 atm (Low) and 5–8 atm (High) as indicated above. The columns represent three experiments, performed in duplicates, in which different polymers described in Fig. 2B were used to increase the osmotic pressure. Error bars are the standard deviation.

strength of submerged biofilms (Hohne *et al.*, 2009) or impeding the growth of the biofilm (Jones *et al.*, 2011). By allowing *B. subtilis* to form biofilms naturally around the force probe of the rheometer, we were able to probe the macroscopic robustness of the developing pellicle over time under conditions similar to those used in traditional, bench-top studies without impeding biofilm development. This approach is different and complementary to previous assays in which large biofilms are first grown and then transported to a rheometer (Jones *et al.*, 2011) for mechanical testing or alternatively are grown within a confined microenvironment. Additionally, by using polymers as our osmotic agents rather than small molecules that are more commonly used (such as salts and sugars), we were able to avoid alternative physiological effects well as distinguish among various possible physical properties such as viscosity and ionic effects. We hope that our approach to studying rheology will prove to be of broad applicability in the biofilm field.

## Experimental procedures

### Strains and media

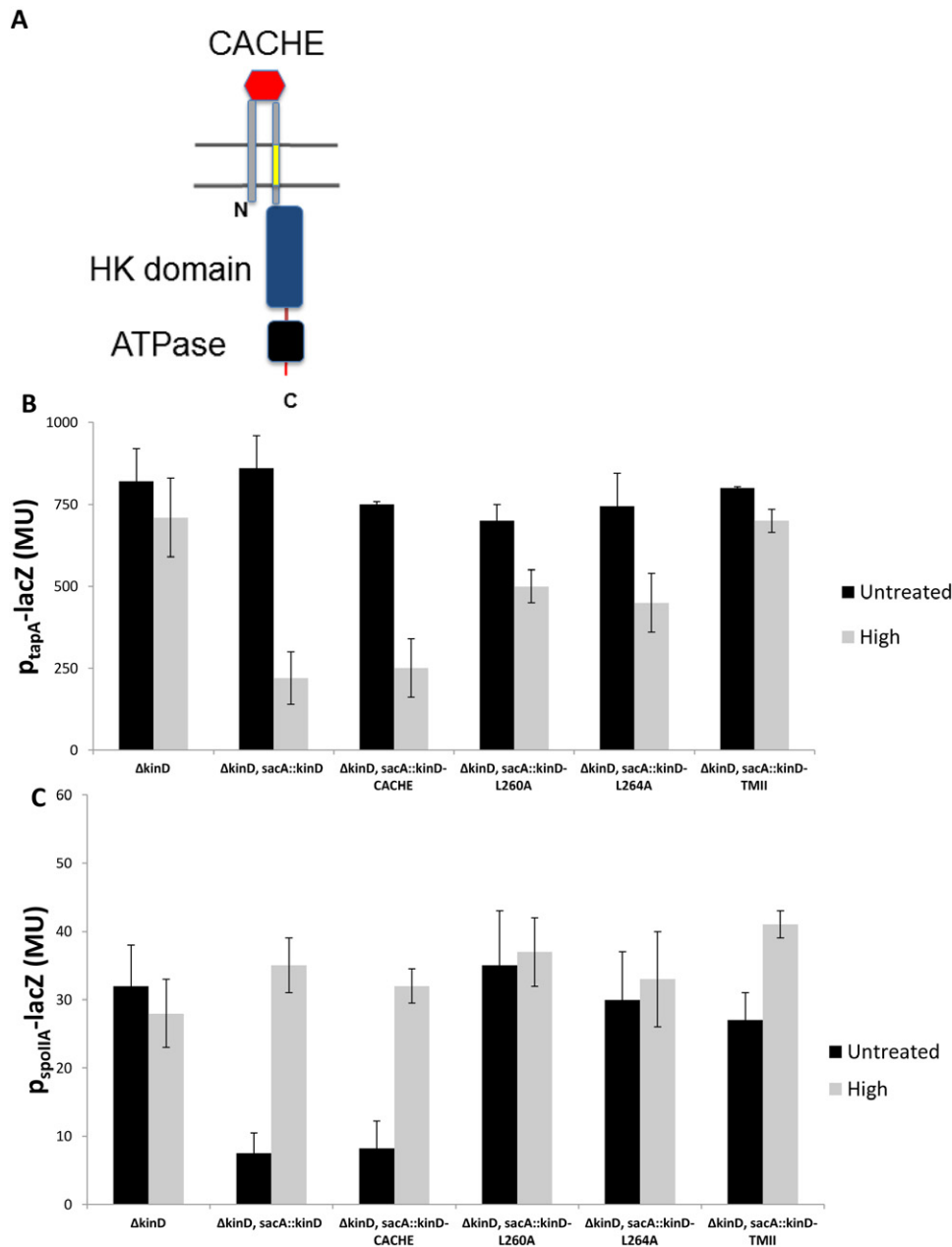
*Bacillus subtilis* NCIB3610 and its derivatives were grown in Luria–Bertani (LB) medium at 37°C or MSgg medium (Branda *et al.*, 2001) at 23°C.

### Strains used in this work

All *B. subtilis* strains are derivatives of NCIB 3610, a wild strain that forms robust biofilms (Branda *et al.*, 2001), and are listed in Table S1.

### Strain construction

Strains were constructed using standard methods (Sambrook and Russell, 2001). SPP1 phage-mediated transduction was used to introduce antibiotic resistance-linked mutations from PY79 derivatives into NCIB3610 in different backgrounds



**Fig. 8.** Amino acid substitutions in a transmembrane segment of KinD impair osmosensing.

A. Transmembrane segment II of KinD, which is similar to the transmembrane segment of EnvZ is highlighted in yellow.

B and C. The activities of  $P_{spolIA-lacZ}$  (B) and  $P_{tapA-lacZ}$  (C) were measured at the indicated atms with derivatives of a mutant deleted for *kinD* (RL4552) and containing the indicated mutant and wild-type alleles of *kinD* at *sacA* (IKG613). *kinD*-CACHE is mutant for the CACHE domain (Chen *et al.*, 2012) (IKG614) and *kinD*-TMII contains the coding sequence for the transmembrane segment of KinB in place of the coding sequence for the second (II) transmembrane domain of KinD (IKG615). High osmolarity was as described for Fig. 7. The columns represent three experiments, performed in duplicates, in which different polymers described in Fig. 2B were used to increase the osmotic pressure. Error bars are the standard deviation.

(Yasbin and Young, 1974). Mutants of *kinD* were created using a QuikChange II XL site-directed mutagenesis kit (Stratagene) and the primers in Table S2.

#### Pellicle formation

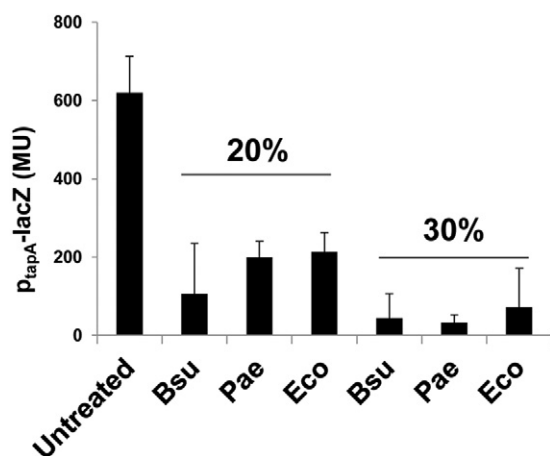
For pellicle formation in liquid medium, cells were grown to exponential phase and 3  $\mu$ l of culture were mixed with 3 ml of

biofilm-inducing medium in a 12-well plate (VWR). Plates were incubated at 22°C. Images of colonies and pellicles were taken using a SPOT camera (Diagnostic Instruments, USA).

#### $\beta$ -Galactosidase assays

Pellicles were formed in MSgg medium (Branda *et al.*, 2001) with/without polymers as indicated in the legend of each figure.





**Fig. 9.** Heterologous EPS inhibits matrix gene expression. An  $\Delta\text{eps}$  mutant containing  $P_{\text{lapA-lacZ}}$  (IKG600) was grown in medium supplemented with either 20% or 30% EPS extracted from *B. subtilis* 3610, *Pseudomonas aeruginosa* PA14 and *E. coli* W3100 pellicles. Per cent EPS was calculated by dry weight in the total solution. The assay was performed in 96-well plates.

The pellicle were collected, washed twice with PBS and sonicated to remove the extracellular matrix.  $\text{OD}_{600}$  had been measured. A total of  $10^8$ – $10^9$  cells were taken for the assay. Cells were spun down and pellets were resuspended in 1 ml of Z buffer (40 mM  $\text{NaH}_2\text{PO}_4$ , 60 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM  $\text{MgSO}_4$ , 10 mM KCl and 38 mM  $\beta$ -mercaptoethanol) supplemented with 200  $\mu\text{g ml}^{-1}$  freshly made lysozyme. Resuspensions were incubated at 30°C for 15 min. Reactions were started by adding 200  $\mu\text{l}$  of 4 mg  $\text{ml}^{-1}$  ONPG (2-nitrophenyl  $\beta$ -D-galactopyranoside) and stopped by adding 500  $\mu\text{l}$  of 1 M  $\text{Na}_2\text{CO}_3$ . The soluble fractions were transferred to cuvettes (VWR), and  $\text{OD}_{420}$  values of the samples were recorded using a Pharmacia ultraspectrometer 2000. The  $\beta$ -galactosidase-specific activity was calculated according to the equation  $[(\text{OD}_{420}/(\text{time} \times \text{OD}_{600})) \times \text{dilution factor} \times 1000]$ . Assays were conducted in duplicate.

#### Osmotic pressure modification

The osmotic pressure in the experimental system was modified by the addition of polymers to the growth medium. Dextran and PEG samples were purchased from Sigma-Aldrich. Several weight fractions of polymer were dissolved in MSgg or LB and left to tumble slowly until the solution turned optically clear. The osmotic pressure of the modified medium was measure with an 'Advanced Instruments Freezing Point Osmometer' (model 3300). The contribution of the polymer to the osmotic pressure was calculated by subtracting the measured osmotic pressure of the pure medium. In addition, by comparing these values with the measurements of the osmotic pressure of the identical mass fractions of polymer dissolved in deionized water we found that the contribution of the polymer to the osmotic pressure was largely identical for all our media. The viscosity of the modified medium was measure on an 'Ares G2' using a double-walled Cuvette Geometry.

#### Pellicle rheology

For the time-lapse measurements (Fig. 1C) pellicles were grown on top of an 'Ares G2' strain controlled rheometer. Fifteen millilitres of MSgg (with or without PEG depending on the experimental intention) were poured into a stainless steel 34 mm well. A cylinder (15 mm diameter) was placed at the centre of the well, 7 mm above the bottom so that the liquid–air interface was at approximately half the cylinders height. The cylinder was coupled to the torque sensor of the rheometer. Repetitive, low strain ( $\leq 1\%$ ) tests of the pellicles strength were conducted by measuring the oscillatory torque on the cylinder,  $\tau$ , while rotating the well around its symmetry axis periodically at small amplitudes ( $\leq 0.6^\circ$ ). In this geometrical configuration the measurements were largely insensitive to the fluid and biomass on which the biofilm was formed. This is due to the existence of a large gap between the inner and outer walls. To verify this we used an identical set-up and measurement parameters to measure the shear response of inoculated LB medium at an optical density ( $\text{OD}_{600}$ ) greater than 0.5. For this sample we did not resolve any signal above the noise level of the instrument. The whole set-up was isolated and held under a controlled temperature of 30°C.

To compare the strength of pellicles grown under different osmotic pressures (Fig. 1D) 6  $\mu\text{l}$  of cells in exponential phase were mixed with 6.5 ml of MSgg modified with appropriate amounts of 20 kDa PEG in six well plates (VWR). Roughened PMMA cylinders (15 mm diameter) were placed in the centre of the wells 1 mm above the bottom. The top of these cylinders consisted of a smaller diameter cylinder (Fig. 1A) that could be mounted on a 'Bohlin Gemini II' stress controlled rheometer without disturbing the sample. The plates were incubated in ambient conditions (23°C). The shear strength was measured after  $\sim 72$  h of incubation. The first step was to measure the oscillatory torque at low strains ( $< 5\%$ ) (red circles in Fig. 1). Then, we continually sheared the pellicles at a strain rate of 1% per second. The torque increased monotonically until at high enough strains the pellicles began to rupture. (We disregarded tests where rupture initiated at one of the walls.) The maximal torque achieved before rupture is defined as the yield torque.

#### EPS extraction

*Escherichia coli* strain W3100 was grown in static M9 glycerol for 4 days. *B. subtilis* strain 3610 was grown in static biofilm-inducing medium for 3 days. *P. aeruginosa* PA14 was grown in static M63 glycerol medium for 7 days. Pellicles were harvested, washed twice in PBS and mildly sonicated. Cells were removed by centrifugation. The supernatant fluid was mixed with cold isopropanol in a 5:1 ratio and incubated in 4°C overnight. Samples were centrifuged at 8000 r.p.m., 4°C for 10 min. Pellets were resuspended in a digestion mix of: 0.1 M  $\text{MgCl}_2$ , 0.1 mg  $\text{ml}^{-1}$  DNase, 0.1 mg  $\text{ml}^{-1}$  RNase solution, mildly sonicated and incubated for 4 h at 37°C. Samples were extracted twice with phenol-chloroform. The aquatic fraction was dialysed for 48 h against DDW using Slide-A-Lyzer Dialysis with 3.5 MWCO (Thermo Fisher).

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