Identification of genes affecting alginate biosynthesis in *Pseudomonas fluorescens* by screening a transposon insertion library

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Abstract

**Background:** Polysaccharides often are necessary components of bacterial biofilms and capsules. Production of these biopolymers constitutes a drain on key components in the central carbon metabolism, but so far little is known concerning if and how the cells divide their resources between cell growth and production of exopolysaccharides. Alginate is an industrially important linear polysaccharide synthesized from fructose 6-phosphate by several bacterial species. The aim of this study was to identify genes that are necessary for obtaining a normal level of alginate production in alginate-producing *Pseudomonas fluorescens*.

**Results:** Polysaccharide biosynthesis is costly, since it utilizes nucleotide sugars and sequesters carbon. Consequently, transcription of the genes necessary for polysaccharide biosynthesis is usually tightly regulated. In this study we used an engineered *P. fluorescens* SBW25 derivative where all genes encoding the proteins needed for biosynthesis of alginate from fructose 6-phosphate and export of the polymer are expressed from inducible *Pm* promoters. In this way we would avoid identification of genes merely involved in regulating the expression of the alginate biosynthetic genes. The engineered strain was subjected to random transposon mutagenesis and a library of about 11500 mutants was screened for strains with altered alginate production. Identified inactivated genes were mainly found to encode proteins involved in metabolic pathways related to uptake and utilization of carbon, nitrogen and phosphor sources, biosynthesis of purine and tryptophan and peptidoglycan recycling.

**Conclusions:** The majority of the identified mutants resulted in diminished alginate biosynthesis while cell yield in most cases were less affected. In some cases, however, a higher final cell yield were measured. The data indicate that when the supplies of fructose 6-phosphate or GTP are diminished, less alginate is produced. This should be taken into account when bacterial strains are designed for industrial polysaccharide production.

**Keywords:** *Pseudomonas fluorescens*, Alginate biosynthesis, Transposon mutants, Fructose 6-phosphate, Purine, Tryptophan, Peptidoglycan recycling

**Background**

Linear polysaccharides composed of mannuronic and guluronic acid residues that may be O-acetylated, are denoted alginate. These polymers are synthesized by brown and some red algae and by bacterial species belonging to the genera *Azotobacter* and *Pseudomonas*. Alginites manufactured from brown algae are currently used in diverse industrial and pharmaceutical applications. However, alginates produced by bacteria can more easily be tailored to obtain the compositions desired for the more high-value end of the alginate market [1], and this has motivated our studies on alginate-producing bacteria.

Production of a secreted polysaccharide imposes a drain on the cell's carbon and energy sources, and thus the biosynthesis is usually tightly regulated under natural conditions. In batch cultures, alginate-producing *P. fluorescens* mutants display a reduced cell yield compared to the corresponding non-alginate producing strains [2]. Bacterial
alginate production is controlled by the alternative sigma factor AlgU and is usually turned off in *Pseudomonas spp.* Induction of alginate biosynthesis results in a proteolytic cascade that finally cleaves the AlgU anti-sigma factor MucA, leading to transcription of the genes in the *alg* operon [3].

In the first steps of bacterial alginate biosynthesis fructose 6-phosphate (Fru6P) is converted to GDP-mannuronic acid by the concerted action of AlgA, AlgC and AlgD. GDP-mannuronic acid is then polymerized to polymannuronic acid by Alg8 and the copolymerase Alg44. Together with AlgG, AlgX, AlgK and AlgE these form a protein complex that transports the alginate out of the cell as depicted in Fig. 1a [4]. AlgG also epimerizes some M-residues to G, while AlgI, AlgL, AlgF and AlgX are needed to O-acetate some of the M-residues. The alginate lyase AlgL removes alginate molecules that have been released to the periplasm [5]. Twelve of the thirteen genes directly involved in alginate biosynthesis are found in the *alg* operon, while the last, *algC*, is found elsewhere on the chromosome. This gene organization is found in all characterized alginate-producing bacteria. In addition to Fru6P and GTP, dimeric cyclic di-GMP (c-di-GMP) is needed for bacterial alginate biosynthesis [6, 7].

Recently we showed that the alginate synthesis rate is not proportional to the number of alginate biosynthetic complexes, indicating that there must be some kind of metabolic control as well [4]. In a recent transposon screen, some genes affecting AlgU-regulation were identified in *P. aeruginosa* [8]. However, the aim of the present study was to identify genes and pathways that influence alginate biosynthesis indirectly by perturbing the cell’s metabolism. An alginate-producing *P. fluorescens* strain in which the *alg* operon and *algC* is under control of the inducible *Pm* promoter was constructed and subjected to transposon mutagenesis. The *Pm* promoter and its activator XylS originally controls expression of the genes of the meta-cleavage pathway of aromatic hydrocarbons on the *Pseudomonas putida* plasmid pWW0 [9]. We have earlier shown that the *Pm* promoter and the weaker *Pm* promoter derivative *Pm-G5* are useful for obtaining different levels of controlled gene expression in *P. fluorescens* [5]. About 11500 insertion mutants were screened with respect to growth and alginate biosynthesis, and the inactivated genes in mutants displaying altered alginate yields were identified. The results supported our hypothesis that further levels of post-translational regulation exist, allowing the cell to prioritize basic cellular metabolism over alginate biosynthesis.

**Results and discussion**

**Construction of a *P. fluorescens* strain in which the alginate biosynthesis genes are controlled by the inducible *Pm* promoter**

In order to avoid re-identification of the genes already known to directly regulate expression of the structural

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**Fig. 1** The relationship between alginate biosynthesis and the cellular metabolism in *P. fluorescens*. **a** The proteins and metabolites needed for alginate biosynthesis, **b** A simplified model of the cell’s metabolism highlighting the processes identified in the present study as being important for full alginate biosynthesis levels. The genes discussed in the paper are highlighted in yellow. The Entner-Doudoroff pathway and the oxidative part of the pentose phosphate pathway are indicated by red arrows, and the non-oxidative part of the pentose phosphate pathway with purple arrows. Green arrows indicate other pathways competing with accumulation of the three metabolites Fru6P, GTP and c-di-GMP, while blue arrows indicate pathways that would increase the synthesis of one of these three metabolites. Each arrow may represent several enzymatic steps. Abbreviations: OM: Outer membrane, IM: Inner membrane, M: Mannuronic acid residue, G: guluronic acid residue, Ac: Acetyl, TCA: Tricarboxylic acid cycle, PP: Peptidoglycan, Trp: Tryptophan, PRPP: Phosphoribosyl pyrophosphate, RSP: Ribose 5-phosphate, E4P: Erythrose 4-phosphate.
alginate biosynthetic genes, a derivative of *P. fluorescens* SBW25 designated strain MS1 was constructed (Fig. 2a). In this strain the naturally regulated *algD* promoter (which controls expression of the *alg* operon) was substituted with the wild-type *Pm* promoter. *xylS*, encoding the activator protein needed for expression from the *Pm* promoter, was inserted upstream of *Pm*. Then *algC* was inactivated by an in-frame deletion followed by a chromosomal insertion of a transposon containing a new *algC* copy expressed from a mutant version of *Pm* (*PmG5*) [5, 10]. This strain, designated MS2, produces only a small amount of alginate in the absence of *Pm* induction due to the low uninduced activity of *PmG5*.

Alginate production has been reported to affect cell yield in *P. fluorescens* [2], and it was also possible that m-toluic acid would have an effect on growth. This was tested by cultivating the non-alginate producing wild type strain SBW25 and strain MS2 in Biolector® for three days in 0.5 x PIA supplemented with glycerol as carbon source. Growth rate and cell yield was significantly lower for the induced strain MS2 relative to the non-alginate producing strain, while no effect was seen by cultivating SBW25 in the presence or absence of 0.5 mM m-toluic acid (Additional file 1: Figure S1).

The transposon carrying *algC* was found to disrupt PFLU2944, which is the last gene in an operon encoding a putative ABC transporter (Fig. 2b). In the presence of the *Pm*/*PmG5* inducer (m-toluate), the alginate production of strain MS2 was similar to that of strain MS1 (results not shown).

### Construction of a transposon insertion library and screening with respect to alginate synthesis

The transposon-containing suicide vector pMS11 (Fig. 2c) was used for mutagenesis of strain MS2. Nearly 11500 insertion mutants were picked robotically from the original agar medium plates and cultivated in 96-deep-well microtiter plates containing 0.5x liquid PIA with glycerol and *m*-tolute. After three days, cell densities and alginate production were measured. The initial screen was followed by a rescreen of primary candidates and 184 mutants were found to produce less than 50% (163 mutants) or more than 110% alginate (21 mutants) when compared to the parent strain. The transposon insertion sites in all these mutants were determined by DNA sequencing, leading to identification of 134 different genes belonging to most of the main cellular functions (results not shown). Of these genes only ten were known alginate biosynthesis structural genes, while one was *xylS*, the positive regulator of *Pm* expression. These results show that about 92% of the identified genes are not directly associated with alginate synthesis. The screen did not cover all relevant genes in the genome, since insertions in *algG*, *algF* and *algI* (members of the *alg* operon) were not found.

### Evaluation of the mutants to select candidates for further studies

Sequenced mutants with altered alginate phenotypes were cultivated in triplicates in 96-deep-well microtiter plates in three different media; 0.5xPIA with glycerol and 0.5xDEF4 with fructose or glycerol as carbon source. Alginate production was measured in the presence or absence of 0.5 mM m-toluic acid. The transposon insertion sites in all these mutants were determined by DNA sequencing, leading to identification of 134 different genes belonging to most of the main cellular functions (results not shown). Of these genes only ten were known alginate biosynthesis structural genes, while one was *xylS*, the positive regulator of *Pm* expression. These results show that about 92% of the identified genes are not directly associated with alginate synthesis. The screen did not cover all relevant genes in the genome, since insertions in *algG*, *algF* and *algI* (members of the *alg* operon) were not found.

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sources (7 g/L), and 0.5 mM m-toluate to induce alginate production. In the DEF4 media ammonium is the only nitrogen source, while PIA contains peptone that may be used as both nitrogen and carbon source. Furthermore, DEF4 contains more phosphate than PIA. The alginate yield from the control strain (MS2) was significantly higher in the DEF4 media, about 3 g/L compared to about 1 g/L in PIA, which resulted in better accuracy of the data in DEF4 for low alginate producers.

Results for mutants displaying significantly altered alginate production levels in at least one of the three media, are shown in Table 1. Significant changes were defined as less than 50% or more than 110% of the alginate production of the parent strain, and 36% of the retested gene-inactivation mutants did not meet this criterion. No mutant produced more alginate than the control strain in all three media. Mutants with insertions in alginate biosynthetic genes and xylS did, as expected, not produce alginate and are not included in Table 1. When several mutants had the same gene inactivated and displayed similar phenotypes, results from only one of them are shown in Table 1. For mutants where genes involved in glycerol utilization, amino acid biosynthesis or phosphate uptake had been inactivated, one would expect that the observed effects on biomass and alginate yield should be media dependent. As shown in Table 1 this was the case for most genes belonging to these categories.

It is probable that in many cases the phenotype observed in a transposon insertion mutant is caused directly by inactivation of the identified gene. However, polar effects (particularly in operons) and unrelated, spontaneous mutations can certainly not be excluded. For those genes where several independent transposon insertion mutants were identified, it is more likely that the observed phenotype is caused by the observed transposon insertion. The same argument may be used when several genes encoding proteins in the same metabolic pathway have been identified. In addition, 18 of the identified genes were chosen to be complemented either by expressing the wild type gene on a transposon or by adding the lacking metabolite.

The transposons were constructed and transferred to the mutant strains, and both the mutant strains and the complemented strains were cultivated in two new growth experiments (Tables 2 and 3). Two of the 18 mutants could not be complemented and are not discussed further. These results show that the phenotypes of 16 out of 18 (89%) tested mutants can be explained by the transposon insertions only.

Alginic acid synthesis requires a functional biosynthetic complex, Fru6P and a dimeric form of c-di-GMP (Fig. 1a). Interestingly, the majority of those mutants that reproducibly produced less alginate were assigned to the groups involved in uptake and metabolism of carbohydrates, amino acids and nucleotides (Table 1). In addition four genes encoding proteins involved in protein modification were identified. Fig. 1b summarizes how the pathways identified in the current study might influence alginate yield, and these genes and pathways are discussed in more detail below.

**Alginic acid production is influenced by signal transduction systems involved in carbon, nitrogen and phosphor metabolism**

Four different signal transduction systems, CbrAB, NtrBC, PTS$^{Ntr}$, and PhoBR, were identified in the screen by using the criteria of either complementation or identification of several independent mutants in specific genes or pathways. The CbrAB two-component system has been described in several species of *Pseudomonas* as sensors and regulators of genes involved in utilization of different carbon and nitrogen sources, and has been proposed as sensors for the C/N balance in the cell [11, 12]. It has been shown that CbrB activates the expression of non-coding RNAs that relieve the catabolite repression otherwise exerted by Crc [13]. In *P. putida*, inactivation of cbrB also affected stress responses and biofilm development [14]. Our results show that the identified cbrB mutant produces less alginate (0-63%) than the otherwise isogenic control strain in all three media (Table 2). The mutant could be complemented by introducing a transposon-encoded copy of cbrB (Table 2). The effect of inactivating cbrA was, however, less pronounced, and might be caused by a polar effect on cbrB (Table 1). In *P. putida*, a cbrB mutant was shown to be unable to use some amino acids as carbon source, and to have an increased expression level of some of the genes encoding proteins involved in the Entner-Doudoroff pathway [14]. If the consequences of inactivating cbrB is similar in *P. fluorescens*, these two effects alone might explain the observed growth and alginate yields for the cbrB mutants, by reducing the net flow to Fru6P (Fig. 1b). However, given the known pleiotropic nature of a cbrB mutation, this probably is not the full explanation.

NtrBC is known to be an important response regulator system for bacterial nitrogen sensing, and has been found to interact with the CbrAB system [14]. GlnE is needed for the posttranscriptional activation of glutamine synthase, which is a part of the NtrC regulatory cascade [15]. It has been shown that inactivation of this gene lowered the pool of Fru6P in *Corynebacterium glutamicum* [16]. Consistent with this the alginate yield was significantly lower in PIA and in DEF4 with fructose for both glnE mutants (Table 1).

Glutamine and α-ketoglutarate are used by the NtrC-cascade to sense the carbon and nitrogen status
| Number of independent transposon mutants | Gene ID | Gene Function | Growth (G) and Alginate production (A) in different media a |
|------------------------------------------|--------|---------------|-------------------------------------------------|
|                                          |        |               | PIA Gly | 0.5xDEF4 Gly | 0.5xDEF4 Fru |
|                                          |        |               | G       | A          | G          | A          | G          | A          |
|                                          |        |               | % SD    | % SD       | % SD       | % SD       | % SD       | % SD       |
| SBW25 WT                                 |        |               | 132     | 6          | 25         | 86         | 0          | 0          |
|                                          |        |               | 100     | 6          | 130        | 6          | 100        | 6          |
| Control                                  |        |               | 100     | 6          | 100        | 6          | 100        | 6          |
| 1                                        |        | aceE1 Energy production and conversion | 66      | 2          | 77         | 4          | 100        | 6          |
| 1                                        |        | aceE2         | 108     | 4          | 60         | 120        | 16         | 4          |
| 1                                        |        | cliB          | 151     | 8          | 86         | 16         | 100        | 6          |
| 2                                        |        | ftsK Cell cycle control, cell division, chromosome partitioning | 38      | 0          | 72         | 4          | 100        | 6          |
|                                          |        |               | 107     | 6          | 100        | 6          | 96         | 8          |
|                                          |        |               | 106     | 6          | 97         | 7          | 100        | 6          |
|                                          |        |               | 104     | 4          | 106        | 6          | 72         | 6          |
|                                          |        | ilvD          | 69      | 2          | 60         | 10         | 17         | 1          |
|                                          |        | trpD          | 127     | 4          | 102        | 8          | 13         | 1          |
|                                          |        | trpE          | 102     | 2          | 105        | 5          | 0          | 0          |
|                                          |        | purH Nucleotide transport and metabolism | 44      | 5          | 81         | 3          | 0          | 0          |
|                                          |        | purF          | 55      | 2          | 81         | 2          | 9          | 6          |
|                                          |        | purL          | 50      | 1          | 10         | 70         | 1          | 3          |
|                                          |        | purK          | 84      | 4          | 113        | 8          | 0          | 4          |
|                                          |        | purE          | 88      | 7          | 87         | 5          | 37         | 5          |
|                                          |        | amn           | 79      | 2          | 106        | 2          | 66         | 9          |
|                                          |        | gkp Carbohydrate transport and metabolism | 52      | 2          | 39         | 19         | 7          | 2          |
|                                          |        | gkpF          | 59      | 2          | 39         | 19         | 7          | 2          |
|                                          |        | paaF          | 109     | 5          | 94         | 4          | 84         | 18         |
|                                          |        | treZ          | 107     | 2          | 12         | 97         | 1          | 98         |
|                                          |        | acnA          | 144     | 8          | 92         | 7          | 68         | 23         |
|                                          |        | pyKA          | 107     | 4          | 14         | 51         | 3          | 132        |
|                                          |        | hemE Coenzyme transport and metabolism | 147     | 5          | 68         | 8          | 98         | 2          |
|                                          |        |               | 152     | 10         | 52         | 16         | 98         | 1          |
|                                          |        |               | 109     | 5          | 94         | 4          | 84         | 18         |
|                                          |        |               | 102     | 10         | 9          | 91         | 12         | 75         |
|                                          |        |               | 114     | 6          | 43         | 6          | 47         | 6          |
|                                          |        |               | 117     | 7          | 58         | 5          | 62         | 3          |
|                                          |        |               | 67      | 1          | 2          | 89         | 2          | 60         |

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Table 1: Identified mutants and their growth yield and alginate production in the three media\(^a\) (Continued)

| Gene   | Description                        | Yield | Alginate |
|--------|------------------------------------|-------|----------|
| PFLU4993 | ampG | 58 | 2.4 | 2.87 | 1.53 | 1.10 | 198.6 | 83.17 |
| PFLU5439 | mpl | 129 | 6.36 | 5.103 | 1.80 | 8.106 | 4.46 | 6.6 |
| PFLU5546 | annK | 62 | 3.8 | 2.99 | 9.9 | 11.204 | 1.65 | 6.6 |
| PFLU5547 | amgK | 54 | 4.3 | 2.86 | 4.68 | 11.192 | 1.59 | 26.21 |
| PFLU5573 | glnE | 95 | 30.45 | 10.23 | 2.15 | 4.192 | 1.18 | 46.38 |
| PFLU561 | ispA | 88 | 5.2 | 6.85 | 4.46 | 8.282 | 3.15 | 7.7 |
| PFLU4418 | fleN | 88 | 1.43 | 4.80 | 4.104 | 29.80 | 1.40 | 12.12 |
| PFLU4439 | fisF | 140 | 8.12 | 2.97 | 13 | 0.342 | 3.10 | 0.0 |
| PFLU4448 | fliC | 111 | 8.38 | 2.87 | 5.102 | 7.171 | 3.132 | 6.6 |
| PFLU0870 | tldD | 111 | 3.26 | 5.103 | 1.88 | 6.137 | 6.111 | 16.1 |
| PFLU2032 | pcc | 100 | 2.37 | 6.81 | 1.59 | 6.124 | 7.26 | 6.6 |
| PFLU2614 | sohB | 98 | 3.11 | 3.96 | 1.73 | 8.108 | 7.109 | 12.19 |
| PFLU3795 | cipA | 147 | 7.60 | 7.104 | 1.127 | 20.256 | 8.124 | 21.13 |
| PFLU4383 | dsbC | 118 | 6.21 | 8.87 | 2.22 | 5.151 | 2.298 | 8.38 | 13.21 |
| PFLU5007 | cipA | 102 | 7.13 | 6.97 | 2.79 | 5.120 | 1.10 | 8.2 |
| PFLU591 | ppX | 50 | 2.82 | 3.38 | 7.67 | 2.90 | 13.76 | 2.6 |
| PFLU5911 | ppX | 54 | 1.0 | 0.73 | 4.101 | 7.107 | 5.98 | 15.11 |
| PFLU5051 | rssA | 108 | 5.86 | 11.96 | 4.101 | 32.104 | 12.124 | 26.1 |
| PFLU2104 | | 149 | 9.66 | 18.94 | 1.65 | 13.117 | 7.136 | 15.117 |
| PFLU2996 | | 134 | 10.66 | 7.104 | 3.74 | 12.122 | 9.132 | 5.13 |
| PFLU3202 | | 108 | 3.76 | 10.81 | 3.47 | 6.403 | 7.23 | 3.13 |
| PFLU3391 | | 51 | 25.58 | 8.68 | 1.95 | 7.118 | 0.129 | 13.21 |
| PFLU3411 | | 105 | 6.50 | 9.101 | 2.70 | 3.135 | 2.137 | 19.1 |
| PFLU3456 | | 104 | 4.47 | 5.103 | 0.107 | 2.151 | 13.117 | 6.13 |
| PFLU1838 | | 69 | 0.0 | 0.74 | 14.66 | 14.712 | 26.8 | 0.0 |
| PFLU1995 | | 149 | 7.85 | 9.104 | 1.97 | 7.158 | 18.113 | 17.13 |
| PFLU4517 | | 129 | 3.82 | 6.96 | 3.28 | 5.377 | 18.2 | 6.13 |
| PFLU5779 | | 39 | 2.47 | 21.104 | 5.4 | 3.175 | 32.18 | 7.18 |
| PFLU2489 | | 93 | 10.120 | 51.69 | 3.75 | 8.324 | 40.23 | 9.13 |
| PFLU5377 | | 107 | 8.8 | 3.97 | 4.76 | 5.145 | 1.92 | 20.13 |
| PFLU2629 | | 107 | 6.9 | 3.106 | 4.84 | 13.300 | 16.106 | 7.13 |
| PFLU3162 | | 122 | 2.77 | 20.76 | 1.88 | 12.280 | 36.96 | 15.13 |
| PFLU3931 | | 97 | 2.35 | 3.75 | 2.3 | 3.327 | 11.12 | 5.13 |
| PFLU2819 | Pseudogene | 114 | 4.148 | 26.102 | 3.98 | 6.337 | 9.89 | 13.13 |
| PFLU5029 | | 89 | 5.7 | 1.101 | 4.70 | 12.127 | 17.101 | 12.13 |
| PFLU0461 | | 85 | 14.10 | 2.120 | 4.104 | 13.317 | 11.67 | 1.13 |
| PFLU4125 | | 119 | 4.164 | 14.112 | 0.86 | 4.158 | 5.72 | 2.13 |
| PFLU5236 | | 140 | 1.34 | 7.105 | 4.55 | 7.319 | 5.66 | 21.13 |
| PFLU5237 | | 118 | 1.15 | 3.109 | 3.23 | 3.483 | 24.31 | 2.13 |
| PFLU5819 | | 80 | 12.8 | 3.90 | 6.79 | 15.49 | 1.5 | 5.13 |
| PFLU6039 | | 78 | 1.44 | 20.102 | 2.90 | 16.110 | 7.84 | 21.13 |
| PFLU6040 | | 25 | 0.0 | 0.97 | 12.87 | 0.99 | 22.100 | 23.13 |
| PFLU2808 | | 100 | 2.11 | 3.110 | 4.75 | 5.255 | 52.19 | 11.13 |
Table 1 Identified mutants and their growth yield and alginate production in the three media (Continued)

|   | Strain  | Description                                      | Yield% | Alginate% | Yield% | Alginate% |
|---|---------|--------------------------------------------------|--------|-----------|--------|-----------|
| 1 | PFLU3002| Intracellular trafficking, secretion, vesicle   | 153    | 6         | 48     | 15        |
|   |         | and transport                                     |        |           |        |           |
| 1 | PFLU3951|                                                   | 106    | 6         | 8      | 105       |
| 1 | PFLU5567|                                                   | 98     | 9         | 61     | 5         |

a: The strains were cultivated in microtiter plates for three days before cell and alginate yield were measured. The mutants shown are those that displayed significantly different alginate production levels in at least one of the three tested media. Data are not shown for strains with transposon insertions in the genes encoded by the alginate operon or in algC. TheTable shows how many independent transposon insertion mutants that were identified for each gene, the gene identifier, the gene name, and which functional group the corresponding protein is assigned to. Growth above 125% and alginate production above 110% are marked using bold types, growth and alginate production between 10 and 50% are marked using italics, and growth and alginate production below 10% are written in bold italics. Three biological replicates were cultivated for each strain, and the results are given as percent (%) of the values obtained from the control strain M2. Standard deviations for the three replicates are shown in the columns to the right (SD).

Inactivation of certain genes involved in cell wall metabolism and vitamin biosynthesis leads to decreased alginate yield

In the present screen, insertions in five of the nine genes known to be involved in peptidoglycan recycling in Pseudomonas [27] were identified as having a negative impact on alginate biosynthesis (mpl, ampG, anmK, amgK and nagZ). The absence of Mpl, which is involved in recycling of the peptide part of peptidoglycan, only slightly decreased the alginate production. However, absence of any of the other four identified enzymes, AmpG, AnmK, AmgK or NagZ, resulted in very low alginate production in the PIA medium and reduced alginate yield in the DEF4 media (Table 1). The sugar phosphates used for peptidoglycan synthesis either originate from peptidoglycan recycling or is synthesized from Fructose (Fig. 1b). Since Fructose is also a precursor for alginate, depletion of this phosphorylated sugar would be expected to cause decreased alginate yield [2]. The nagZ and anmK genes were cloned on transposons, and shown to complement the deficiency in alginate production in the corresponding insertion mutants (Table 2).
| Inactivated gene | Supplement/comp-lement- ing gene (s) | PIA Growth | Algin- ate | 0.5xDEF4 Glycerol Growth | Algin- ate | 0.5xDEF4 Fructose Growth | Algin- ate |
|-----------------|---------------------------------|------------|----------|--------------------------|----------|--------------------------|----------|
| wt              |                                 | 100        | 100      | 100                      | 100      | 100                      | 100      |
| trpF            |                                 | 65         | 0        | 145                      | 0        | 291                      | 38       |
| tryptophane     |                                 | 70         | 139      | 78                       | 60       | 130                      | 45       |
| trpD            |                                 | 56         | 0        | 1                        | 0        | 3                        | 0        |
| tryptophane     |                                 | 68         | 261      | 68                       | 80       | 71                       | 75       |
| trpD            |                                 | 85         | 142      | 114                      | 23       | 242                      | 38       |
| trpDC           |                                 | 89         | 85       | 81                       | 91       | 152                      | 120      |
| purH            | Adenine, thiamine               | 51         | 188      | 14                       | 33       | 39                       | 21       |
| purH            |                                 | 90         | 100      | 91                       | 88       | 163                      | 97       |
| purE            | Adenine, thiamine               | 56         | 91       | 17                       | 50       | 43                       | 29       |
| purE            |                                 | 57         | 0        | 1                        | 0        | 1                        | 0        |
| purL            | Adenine, thiamine               | 52         | 124      | 12                       | 48       | 38                       | 30       |
| ilvD            |                                 | 77         | 18       | 2                        | 2        | 4                        | 0        |
| ilvD            |                                 | 91         | 82       | 91                       | 109      | 222                      | 97       |
| aceEI           |                                 | 33         | 58       | 6                        | 20       | 13                       | 7        |
| aceEI           |                                 | 96         | 42       | 85                       | 96       | 244                      | 123      |
| PFLU3030        |                                 | 88         | 0        | 80                       | 89       | 104                      | 103      |
| PFLU3030        |                                 | 97         | 142      | 89                       | 123      | 101                      | 111      |
| dsbC            |                                 | 108        | 52       | 111                      | 89       | 149                      | 115      |
| dsbC            |                                 | 105        | 127      | 105                      | 117      | 94                       | 92       |
| sohB            |                                 | 100        | 9        | 100                      | 50       | 479                      | –2       |
| sohB            |                                 | 91         | 109      | 104                      | 106      | 109                      | 100      |
| nagZ            |                                 | 53         | 0        | 139                      | 93       | 115                      | 75       |
| nagZ            |                                 | 87         | 145      | 84                       | 120      | 289                      | 79       |
| anmK            |                                 | 32         | 0        | 124                      | 98       | 197                      | 99       |
| anmK            |                                 | 84         | 118      | 86                       | 135      | 111                      | 128      |
| ispA            |                                 | 82         | 0        | 100                      | 97       | 121                      | 115      |
| ispA            |                                 | 92         | 212      | 88                       | 117      | 405                      | 77       |
| cbrB            |                                 | 83         | 0        | 110                      | 63       | 374                      | 46       |
| cbrB            |                                 | 91         | 118      | 91                       | 95       | 213                      | 107      |
| PFLU3887        |                                 | 91         | 67       | 98                       | 103      | 105                      | 110      |
| PFLU3887        |                                 | 90         | 33       | 109                      | 103      | 112                      | 103      |
| PFLU5567        |                                 | 87         | 64       | 2                        | 6        | 141                      | 30       |
| PFLU5567        |                                 | 93         | 103      | 1                        | 16       | 6                        | 10       |

a: The strains were grown in deep-well plates containing the indicated media for four days before cell and alginate yield were measured. b: empty field denotes no supplement or complementing vector. c: Values are given as percentage of the control strain (SBW25 ΔalgC::TnKB61). Actual values for the control strain were (growth [OD 660]/alginate [g/L]): PIA: 0.492/0.33, DEF4 glycerol: 0.850/1.72, DEF4 fructose: 0.308/3.08. Growth above 125% and alginate production above 110% are marked using bold types, growth and alginate production between 10 and 50% are marked using italics, and growth and alginate production below 10% are written in bold italics.
Three of the identified genes, *aceE1*, *ilvD* and *ispA* were linked to pyruvate metabolism (Fig. 1b). *aceE1* encodes a component of pyruvate dehydrogenase, which is an essential part of the central carbon metabolism. The viability of this mutant might be explained by the presence of other genes encoding AceE-like proteins in *P. fluorescens*. However, the *aceE1* mutant grew more slowly than strain MS2, and hardly produced any alginate. *ilvD* encodes a dihydroxy-acid dehydratase that participates in the biosynthesis of branched amino acids and in the biosynthesis of pantothenate (vitamin B5) and coenzyme A. The *ilvD* mutant displayed a similar phenotype as the *aceE1* strain in all three media (Table 2). The *ispA* mutant would be expected to have defects in the biosynthesis of isoprenoids, which would affect the biosynthesis of ubiquinone and the cell membrane. This mutant produced very low amounts of alginate when grown in PIA, while the phenotypes in the DEF4 media were more similar to the control strain (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2). All three mutants were complemented when the corresponding wild type genes were expressed from (Table 2).

Disruption of several genes encoding proteins involved in protein folding and modification result in reduced alginate yield

Prc is a protease known to affect alginate biosynthesis in some *mucA* mutants of *P. aeruginosa*, and has been proposed to indirectly participate in alginate biosynthetic gene activation through MucA cleavage induced by cell wall stress [30, 31]. However, in our strain both *algC* and the *alg* operon are controlled by the *PmA* promoter, not by the endogenous AlgU-MucA-regulated promoters. Still, four independent *prc* mutants were identified as displaying a reduced alginate yield (Table 1). Our results therefore show that in *P. fluorescens* a *prc* mutation negatively affects alginate biosynthesis even in a *mucA*’ strain. In addition the screen identified another peptidase belonging to the same family, *SohB*, which

### Table 3 Effect of PhoBR disruptions on *P. fluorescens* growth and alginate biosynthesis

| Strain       | Growth (OD600) | Alginate (g/l) |
|--------------|----------------|---------------|
| SBW25mucAHE230 | 2.5+/−0.24     | 4.3+/−0.89    |
| SBW25mucAHE230 ΔphoR | 2.7+/−0.33     | 3.6+/−0.36    |
| SBW25mucAHE230 ΔphoB | 1.4+/−0.12     | 0.0+/−0.0     |
| SBW25mucAHE230 ΔphoR: TnTK5 | 2.4+/−0.09    | 4.0+/−0.24    |
| SBW25mucAHE230 ΔphoR: TnTK7 | 2.1+/−0.10     | 4.3+/−0.66    |
| SBW25mucAHE230 ΔphoB: TnTK6 | 1.5+/−0.20     | 1.2+/−0.56    |
| SBW25mucAHE230 ΔphoR: TnTK7 | 2.0+/−0.13     | 5.5+/−0.12    |

a: The cells were grown for 72 h in shaking flasks using DEF3 medium with 20 g/l glycerol, 1 μM phosphate and 0.5 mM m-toluolate. Average values from three independent experiments are shown.
also negatively affected alginate yield when inactivated. This phenotype was complemented by a transposon expressing sohB (Table 2). It is unknown which proteins, apart from MucA, is the target of these two proteinases in *P. fluorescens*.

Two genes encoding proteins involved in protein folding were identified in the screen as producing less alginate (Table 1). PFLU4383 encodes a putative thiol-disulfide interchange protein and is located upstream of and partly overlapping *dsbG*, encoding another disulfide isomerase. Three independent inactivations of PFLU4383 were identified. PFLU5007 encodes the disulfide isomerase DsbC and its phenotype was complemented by a transposon-encoded copy of the gene (Table 2). A mutant of *P. aeruginosa* with transposon-inactivated *dsbC* was recently found to display a non-mucoid phenotype [32], indicating that DsbC is needed for normal levels of alginate production in both species. The results suggest that full alginate production in these media depend on correct folding of some proteins. It remains unknown which proteins need these isomerases for correct folding.

**Conclusion**

In an earlier study, it was shown that inactivation of glucose-6-phosphate dehydrogenase increased alginate yield when glycerol was used as carbon source, and this indicated that the availability of Fru6P may be one limiting factor to sustain high level alginate production [2]. Furthermore, it has been shown that the number of alginate biosynthetic complexes are not influenced by the absence of precursors for alginate synthesis [4], indicating that these complexes are not destabilized in the absence of polymer synthesis. The aim of screening a transposon insertion library, was to discover genes and metabolic pathways that indirectly influence alginate production in *P. fluorescens*. The main conclusion of our data is that alginate biosynthesis depends on sufficient levels of Fru6P, GTP and c-di-GMP (Fig. 1b). Inactivation of genes in several systems sensing the carbon/nitrogen ratio resulted in mutants that produce less alginate than the parent strain, and this further indicates that alginate production might be down-regulated as a response to a perceived carbon limitation. A majority of the analysed mutants displayed a significantly decreased alginate yield, while the cell yield was less affected, and in some cases even increased. This suggests that when *P. fluorescens* is facing certain nutrient limitations, less alginate is produced.

**Methods**

**Growth of bacteria**

*E. coli* and *P. fluorescens* (Table 4) were routinely cultivated in L broth or on L agar at 37 °C or 30 °C, respectively [33]. *P. fluorescens* was also grown in PIA medium [33], DEF4 medium [34] and DEF3 medium with low phosphate: KH₂PO₄ 0.14 mg/L, KCl 0.36 g/L, NH₄Cl 2.21 g/L, citric acid·H₂O 0.9 g/L, ferric citrate 0.02 g/L, H₃BO₃ 0.001 g/L, MnCl₂·4H₂O 0.005 g/L, EDTA·2H₂O 0.0039 g/L, CuCl₂·2H₂O 0.0005 g/L, Na₂MoO₄·2H₂O 0.0008 g/L, CoCl₂·6H₂O 0.0008 g/L, Zn (CH₃COO)₂·2H₂O 0.0027 g/L, NaCl 1.56 g/L, MgSO₄·7H₂O 0.57 g/L, MOPS 10 g/L. For precultures, 0.39 g/L yeast extract was added to the DEF4 medium. The pH of DEF3 and DEF4 was adjusted to 7.0. Carbon sources – fructose or glycerol – were added to 20 g/L. Antibiotics used: ampicillin (Ap, 200 mg/L), tetracycline (Tc, 15 mg/L), apramycin (Am, 25 mg/L), kanamycin (Km, 50 mg/L). For growth in microtiter plates and micro bioreactors (BioLector®), half the concentrations of the media containing 7 g/L carbon source was used, and the cultures were incubated at 25 °C as detailed previously [34]. For some experiments adenine (0.8 mM), thiamine (0.05 mM), or tryptophan (2.5 mM) were added as medium supplements. For growth studies in Biolector® microreactors the cultivations were performed in M2P-labs FlowerPlate® BOH with 1 ml medium per reactor. The cultivations were started (3 vol-% inoculum) from L broth precultures cultivated at 30 °C for 18 h. The BOH plates were incubated at 25 °C, 1300 rpm with 3 mm orbital movement at 80% humidity. pH, dissolved oxygen and biomass were measured automatically every hour by the Biolector system. The biomass measured by the Biolectors Photomultiplier was calibrated by offline optical density measurements using a standard spectrophotometer.

**Analyses of alginate and growth**

The cultures were incubated for three to four days before the cell density and alginate yield were assayed. Enzymatic measurements of alginate production were performed as described earlier [2, 35]. Briefly, the cell free medium were treated with a mixture of an M-specific and a G-specific alginate lyase, and OD₂₃₀ before and after the reaction were measured using a Beckman Coulter robotic liquid handling work station with a Paradigm microplate reader.

**Construction of the transposon vector and the transposon insertion library**

Cloning, transformation, conjugation and gene deletions were performed as described earlier [33]. The plasmids and transposons used and constructed in this study are described in Table 4, while the primer sequences are found in Additional file 2: Table S1. PCR was performed using the Expand High Fidelity kit (Roche). PCR-amplified genes were confirmed by sequencing. Transposon insertions were to be identified by sequencing, so
Table 4 Bacterial strains and plasmids used in this study

| Strains                  | Description                                                                                           | Reference |
|-------------------------|--------------------------------------------------------------------------------------------------------|-----------|
| E. coli S17-1 (λpir)    | λpir (for replication of oriR6K-plasmids) recA, thi pro hsdR-M* RP4 2-Tc:Mu-Km::Tn7PspRSR               | [36]      |
| P. fluorescens SBW25    | Non-mucoid P. fluorescens wild type                                                                    | [37]      |
| SBW25S1                 | Derivative of SBW25 where the Pm promoter is inserted directly upstream of algD using pmS9.           | This study|
| SBW25S1 ΔalgC::TnKB61   | Derivative of SBW25 S1 where algC has been deleted utilizing pKB22, and a copy of algC controlled by the PmG5 promoter has been introduced on a transposon inserted into gene PFLU2944. | This study|
| SBW25mucAHE230          | Alginate-producing derivative of SBW25 encoding a defect MucA and where the expression of algC is controlled by the PmG5 promoter | H. Ertesvåg, unpublished |
| SBW25mucAHE230ΔphoB     | Derivative of SBW25mucAHE230 where an in-frame deletion in phoB was introduced utilizing pTK10.       | This study|
| SBW25mucAHE230ΔphoR     | Derivative of SBW25mucAHE230 where an in-frame deletion in phoR was introduced utilizing pTK9.       | This study|

**Plasmids**

| pKD20                   | pUT based transposon vector containing PmG5. Ap', Km'.                                                | [5]       |
| pLitmus28Tc             | High copy number cloning vector. Tc', Ap'                                                             | [5]       |
| pMG48                   | RK2-based gene replacement vector. lacZ+, Tc', Ap'                                                    | [33]      |
| pMC1                    | RK2-based gene replacement vector for replacing the DNA sequence upstream of algD with the Pm-promoter. lacZ+, Tc', Ap' | [10]      |
| pKB22                   | Gene replacement vector for creating an algC-deletion. lacZ+, Tc', Ap'                                 | [5]       |
| pKB60                   | Transposon vector. Contains the transposon TnKB60 with algC under the control of PmG5. Ap', Km'       | [5]       |
| pYQ1                    | pUT based transposon vector containing PmG5. Am', Km'.                                                 | [2]       |
| pEM1                    | Derivative of pLitmus28Tc containing part of the transposon from pKD20. Ap'                            | [2]       |
| pKB61                   | Derivative of pKD60 where a 1.7 kb Avrll-Ncol DNA fragment encoding Km' and most of XylS was exchanged with a 2.5 kb Avrll-Nnot DNA fragment containing tetAR. Tc', Ap' | This study|
| pMS9                    | Derivative of pMC1 where a 0.7 kb SbfII-Nnot DNA fragment containing a gene upstream of algD was exchanged with a PCR product containing the 0.8 kb sequence directly upstream of algD. lacZ+, Tc', Ap' | This study|
| pMS2                    | Derivative of pLitmus28Tc where the tetAR genes were exchanged with a 3.4 kb BamHI-fragment from pKD20 containing the minitransposon and oriR6K. Km', Ap' | This study|
| pMS10                   | Derivative of pMS2 where a 0.5 kb BsiWI-EcoRI-fragment containing oriR6K was deleted and the 1.9 kb NotI-PstI fragment encoding XylS was exchanged with a 0.4 kb PCR product encoding oriR6K. Km', Ap' | This study|
| pMS11                   | Derivative of pKD20 where a 3.7 kb BssHII-SfiI-fragment was exchanged with a 1.5 kb BssHII-SfiI-fragment containing oriR6K from pMS10. Km', Ap' | This study|
| pTK1                    | Derivative of pEM1 in which a 2.2 kb PCR-amplified Ndel-NotI DNA fragment encoding phoBR from P. fluorescens was inserted. Ap' | This study|
| pTK3                    | Derivative of pTK1 in which an inserted 2.2 kb PCR-amplified Ncol-PspOM1 DNA fragment from P. fluorescens including the first 46 nt of phoB replaced most of the phoB gene. Ap' | This study|
| pTK4                    | Derivative of pTK1 from which a 0.9 kb BstEII-BsaBI DNA fragment encoding most of phoR was deleted. Ap'. | This study|
| pTK5                    | Derivative of pKD20 in which a 1.5 kb Ndel-NotI PCR fragment from P. fluorescens containing phoR was inserted. Km'. | This study|
| pTK6                    | Derivative of pKD20 in which a 1.1 kb Ncol-NotI DNA fragment from pTK4 containing phoB8 was inserted. Km'. | This study|
| pTK7                    | Derivative of pKD20 in which a 2.2 kb Ndel-NotI PCR fragment from P. fluorescens containing phoBR was inserted. Km'. | This study|
| pTK8                    | Derivative of pTK1 from which a 0.3 kb BstEII DNA fragment was deleted, creating an in-frame deletion in phoR. Ap'. | This study|
| pTK9                    | Derivative of pMG48 in which a 3.0 kb Ncol-NotI DNA fragment from pTK8 was inserted, containing a deletion in phoR. Ap', Tc'. | This study|
| pTK10                   | Derivative of pMG48 in which a 3.1 kb Ncol-NotI DNA fragment from pTK3 was inserted, containing a deletion in phoB. Ap', Tc'. | This study|
| pYQ1 trpF               | Derivative of pYQ1 in which a 0.7 kb Ndel-NotI PCR fragment encoding TrpF was inserted. Am'. | This study|
| pYQ1 trpD               | Derivative of pYQ1 in which a 1.1 kb Ndel-NotI PCR fragment encoding TrpD was inserted. Am'. | This study|
| pYQ1 trpDC              | Derivative of pYQ1 in which a 1.9 kb Ndel-NotI PCR fragment encoding TrpDC was inserted. Am'. | This study|
Table 4 Bacterial strains* and plasmids used in this study (Continued)

| Strain | Description | Source |
|--------|-------------|--------|
| pYQ1 purH | Derivative of pYQ1 in which a 1.6 kb NdeI-NotI PCR fragment encoding PurH was inserted. Amr. | This study |
| pYQ1 purE | Derivative of pYQ1 in which a 0.5 kb NdeI-NotI PCR fragment encoding PurE was inserted. Amr. | This study |
| pYQ1 ilvD | Derivative of pYQ1 in which a 2.1 kb NdeI-NotI PCR fragment encoding IlvD was inserted. Amr. | This study |
| pYQ1 aceE1 | Derivative of pYQ1 in which a 2.7 kb NdeI-NotI PCR fragment encoding AceE1 was inserted. Amr. | This study |
| pYQ1 PFLU3030 | Derivative of pYQ1 in which a 1.0 kb NdeI-NotI PCR fragment encoding PFLU3030 was inserted. Amr. | This study |
| pYQ1 PFLU3887 | Derivative of pYQ1 in which a 1.0 kb NdeI-NotI PCR fragment encoding PFLU3887 was inserted. Amr. | This study |
| pYQ1 PFLU5567 | Derivative of pYQ1 in which a 1.2 kb NdeI-NotI PCR fragment encoding PFLU5567 was inserted. Amr. | This study |
| pYQ1 dsbC | Derivative of pYQ1 in which a 0.9 kb NdeI-NotI PCR fragment encoding DsbC was inserted. Amr. | This study |
| pYQ1 sohB | Derivative of pYQ1 in which a 1.1 kb NdeI-NotI PCR fragment encoding SohB was inserted. Amr. | This study |
| pYQ1 nagZ | Derivative of pYQ1 in which a 1.1 kb NdeI-NotI PCR fragment encoding NagZ was inserted. Amr. | This study |
| pYQ1 anmK | Derivative of pYQ1 in which a 1.5 kb NdeI-NotI PCR fragment encoding AnmK was inserted. Amr. | This study |
| pYQ1 ispA | Derivative of pYQ1 in which a 0.9 kb NdeI-NotI PCR fragment encoding IspA was inserted. Amr. | This study |
| pYQ1 cbrB | Derivative of pYQ1 in which a 1.4 kb NdeI-NotI PCR fragment encoding CbrB was inserted. Amr. | This study |

* Mutant strains complemented with transposons are not included in the Table

Identification of transposon insertion sites
Genomic DNA was isolated from mutants of interest. For some mutants, the transposon insertion site was identified by direct sequencing using this DNA as the template and the primer MS11 Ori (Additional Table S1). For sequencing on genomic DNA, 5 µg DNA, 50 pmol sequencing primer, 8 µl 2.5x BigDye Terminator Ready Reaction Mix v1.1 (Applied Biosystems) and water to 20 µl was mixed. The reaction was subjected to sixty cycles of 30 s denaturation at 95 °C, 30 s annealing at 52 °C, and four minutes elongation at 60 °C. Alternatively, the DNA flanking the transposon insertion site was cloned by restricting genomic DNA isolated from a transposon mutant with SacI or EcoRI. The fragments were circularized by ligation, and the ligation mixture was transformed into E. coli S17-1 λpir and selected for resistance to kanamycin. Sequencing the resulting plasmids provided better quality sequences than by sequencing directly on genomic DNA. The transposon insertion points were identified by comparing the obtained sequences to the genome sequence (GenBank Accession number AM181176).

Additional file

Additional file 1: Figure S1. Growth profiles of Pseudomonas fluorescence SBW25 and MS2 cultivated in 0.5 x PIA. (PPTX 75 kb)

Additional file 2: Table S1. Primers used in the study. (XLS 30 kb)

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and in the Additional file 2: Table S1 and Additional file 1: Figure S1.

Authors’ contributions
HE supervised the strain and library construction and annotated the mutants to functions. MS constructed the strain, transposon vector and library. HS and GK designed, developed and validated the screening protocols used for analyses and verification of mutant phenotypes. MS, GK and HS participated in the transposon screen, YQS identified inactivated genes and complemented some mutants, TK identified and complemented the phoB8 mutants. HE, SV, HS and TE participated in the initiation and design of the study and in the writing of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.
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