Nanomolar Responsiveness of Marine *Phaeobacter inhibens* DSM 17395 toward Carbohydrates and Amino Acids

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**Abstract**

*Phaeobacter inhibens* DSM 17395 is a heterotrophic member of the ubiquitous, marine *Roseobacter* group and specializes in the aerobic utilization of carbohydrates and amino acids via pathways widespread among roseobacters. The in vivo responsiveness of *P. inhibens* DSM 17395 was studied with nonadapted cells (succinate-grown), which were exposed to a single pulse (100–0.01 µM) each of *N*-acetylglucosamine, mannitol, xylose, leucine, phenylalanine, or tryptophan (effectors). Responsiveness was then determined by time-resolved transcript analyses (quantitative reverse transcription-PCR) of “degradation” and “uptake” genes selected based on previously reported substrate-specific proteome profiles. The transcriptional response thresholds were: 50–100 nM for *nagK* (*N*-acetylglucosamine kinase), *paaA* (ring 1,2-phenylacetyl-CoA epoxidase), and *kynA* (tryptophan 2,3-dioxygenase), 10–50 nM for *xylA* (xylose isomerase), and around 10 nM for *mtlK* (mannitol 2-dehydrogenase). A threshold for leucine could not be determined due to the elevated intrinsic presence of leucine in the exometabolome of succinate-grown cells (no effector addition). Notably, the response thresholds for presumptive carbohydrate-binding proteins of ABC-transporters were in the same range or even lower: 0.1–1 µM for *c27930* (*N*-acetylglucosamine) and even below 10 nM for *c13210* (mannitol) and *xylF* (xylose). These results shed new light on the sensory/regulatory sensitivity of a well-studied roseobacter for recognizing potential substrates at low ambient concentrations and on the concentration threshold below which these might escape biodegradation ("emergent recalcitrance" concept of dissolved organic matter persistence).

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Introduction

The carbon cycle in the oceanic water bodies is to a large part governed by the interplay between phytoplankton and the microbial loop, in particular aerobic heterotrophic microorganisms that degrade (dissolved) organic matter (DOM) arising from primary production. The multistep degradation requires these heterotrophs amongst others to extracellularly decompose biomacromolecules, import liberated mono- and oligomers, and intracellularly metabolize them. This process occurs on a large scale in association with algal blooms and involves members of the flavobacteria (phylum Bacteroidetes) and roseobacters (phylum Proteobacteria) (for overview, see [Buchan et al., 2014; Arnosti et al., 2021]). Furthermore, the activity of such heterotrophs contributes to the shaping of DOM along opposing gradients of increasing molecular diversity and declining substrate concentration (“emergent recalcitrance” concept) [Dittmar et al., 2021].

Roseobacters are globally widespread in temperate and polar oceans, occupy a wide range of habitats, interact with algae, and are metabolically versatile (for overview, see [Buchan et al., 2005; Wagner-Döbler and Biebl, 2006]). Recent whole-genome-based phylogenetic analyses suggest the Roseobacter clade/group be classified as the new family Roseobacteraceae [Liang et al., 2021]. The evolution of roseobacters was studied by comparative genomics, revealing mechanisms of ecological diversification [Luo and Moran, 2014], adaptations to marine habitats [Simon et al., 2017] and corroborating their nutritional diversity and declining substrate concentration. (iii) The genome of P. inhibens DSM 17395 was studied in our laboratory on the physiological/proteogenomic level with respect to differential substrate utilization from complex substrate mixtures [Zech et al., 2013], degradation networks, growth energetics, and metabolic efficiency for amino acids and carbohydrates [Drüppel et al., 2014; Wiegmann et al., 2014; Wünsch et al., 2019], as well as the stoichiometric constraints with respect to varying N/P-provision [Trautwein et al., 2017] and a multifaceted strategy to secure external nitrogen [Trautwein et al., 2018].

The present study investigates the in vivo responsiveness to P. inhibens DSM 17395 for selected carbohydrates (N-acetylglucosamine, mannitol, and xylose) and amino acids (leucine, phenylalanine, and tryptophan) by means of time-resolved, targeted transcript analyses. The degradation routes for these substrates were previously elucidated [Drüppel et al., 2014; Wiegmann et al., 2014] and are schemed together with the coding genes and assigned carbohydrate uptake systems in Figure 1. The aim of this study was to determine the transcriptional response thresholds of P. inhibens DSM 17395 for these substrates and thereby to obtain principle insights about the viability of a marine model heterotroph at low substrate availability and the role of such thresholds within the framework of the “emergent recalcitrance” concept for the persistence of marine DOM.

Phaeobacter inhibens DSM 17395 (formerly Phaeobacter gallaeciensis DSM 17395 [Buddruhs et al., 2013]) is a well-studied heterotrophic model roseobacter with several research foci pursued, as exemplified in the following. (i) P. inhibens DSM 17395 can switch its interaction with the marine microalga Emiliania huxlei from growth promoting via antibiotic (tropodithietic acid, TDA) and auxin secretion to pathogenic via secretion of algacidal compounds (leucine, phenylalanine, and tryptophan) by means of ABC-transporters related to the uptake of the tested carbohydrates. (ii) Biofilm formation by P. inhibens DSM 17395 is a trait required for its aggregation to typical rosette-like structures and attachment to eukaryotic hosts, relying on plasmid encoded functions and potentially involving quorum sensing [Segev et al., 2015; Michael et al., 2016; Beyersmann et al., 2017]. (iii) The genome of P. inhibens DSM 17395 harbors three extrachromosomal elements [Thole et al., 2012], with the large 262-kb plasmid (chromid) harboring the genes for TDA synthesis. Deletion of this plasmid revealed its high energetic burden (∼50% of dissipatory expenditure) and resulted in global responses revealing the functional interconnectivity of the replicons [Trautwein et al., 2016; Wünsch et al., 2020]. (iv) The metabolism of P. inhibens DSM 17395 was studied in our laboratory on the physiological/proteogenomic level with respect to differential substrate utilization from complex substrate mixtures [Zech et al., 2013], degradation networks, growth energetics, and metabolic efficiency for amino acids and carbohydrates [Drüppel et al., 2014; Wiegmann et al., 2014; Wünsch et al., 2019], as well as the stoichiometric constraints with respect to varying N/P-provision [Trautwein et al., 2017] and a multifaceted strategy to secure external nitrogen [Trautwein et al., 2018].

The present study investigates the in vivo responsiveness to P. inhibens DSM 17395 for selected carbohydrates (N-acetylglucosamine, mannitol, and xylose) and amino acids (leucine, phenylalanine, and tryptophan) by means of time-resolved, targeted transcript analyses. The degradation routes for these substrates were previously elucidated [Drüppel et al., 2014; Wiegmann et al., 2014] and are schemed together with the coding genes and assigned carbohydrate uptake systems in Figure 1. The aim of this study was to determine the transcriptional response thresholds of P. inhibens DSM 17395 for these substrates and thereby to obtain principle insights about the viability of a marine model heterotroph at low substrate availability and the role of such thresholds within the framework of the “emergent recalcitrance” concept for the persistence of marine DOM.

Fig. 1. Metabolic and genomic context of genes selected for quantitative transcript profiling of P. inhibens DSM 17395 in response to defined substrate pulses. a. Periplasmic solute-binding proteins of ABC-transporters related to the uptake of the tested carbohydrates. b. Degradation network and (k) coding genes for the degradation of the tested organic substrates; modified from Drüppel et al. [2014] and Wiegmann et al. [2014]. Accession numbers of targeted genes are as followed: nagK, PGA1_c27910; mtlK, PGA1_c13160; xylA, PGA1_c14000; mccB, PGA1_c10320; paaA, PGA1_c04080; kynA, PGA1_c09970; c27930, PGA1_c27930; c13210; and xylF, PGA1_262p00430.

(For figure see next page.)
The diagram illustrates the metabolic pathways for N-Acetylglucosamine (Nag), Mannitol (Man), and Xylose (Xyl). The pathways are detailed with specific gene annotations and reactions, indicating the flow of metabolic processes. Each pathway is marked with relevant genes and reaction steps, showing the integration of these pathways within the organism. The legend at the bottom of the diagram provides a color code for gene functions, distinguishing between metabolic, sensory, regulatory, and transport systems.
Results and Discussion

Substrate (Effector) and Target Gene Selection

For the present study, growth-supporting carbohydrates and amino acids were selected as effectors that are metabolized by *P. inhibens* DSM 17395 via distinct substrate-specific degradation routes widespread among roboseobacters [Drüppel et al., 2014; Wiegmann et al., 2014] and that differ by their general properties and functions: N-acetylglucosamine (C\(_{8}\)H\(_{15}\)NO\(_{6}\)) is an amide of glucosamine and acetate, and represents the monomer of the globally abundant biopolymer chitin (occurring e.g., in fungal cell walls and exoskeleton of mollusks) [e.g., Gooday et al., 1991] and a major building block of peptidoglycan in bacterial cell walls. Mannitol (C\(_{6}\)H\(_{14}\)O\(_{6}\)) is a sugar alcohol that is widespread in algae, serving various functions such as carbon storage and protection against environmental stress [Tonon et al., 2017]. Xylose (C\(_{5}\)H\(_{10}\)O\(_{5}\)) is an aldopentose and a constituent of various natural polysaccharides, e.g., the hemicellulose xylan [Kundu et al., 2019]; its degradation is relevant for biomass-based biorefineries [Basen and Kurrer, 2021; Domingues et al., 2021] and its formation is essential for nucleotide biosynthesis [Jensen et al., 2008]. Leucine (C\(_{6}\)H\(_{13}\)NO\(_{2}\)) belongs to the amino acids carrying aliphatic side chains and to the most abundant ones in proteins; relative abundances of amino acids in proteomes of various organisms have previously been reported [e.g., Hormoz, 2013; Moura et al., 2013]. Phenylalanine (C\(_{9}\)H\(_{11}\)NO\(_{2}\)) is an aromatic amino acid with medium abundance in proteins and an important precursor in the biosynthesis of TDA by *P. inhibens* DSM 17395 [Teufel et al., 2011; Brock et al., 2014]. Tryptophan (C\(_{11}\)H\(_{12}\)N\(_{2}\)O\(_{2}\)) carries an aromatic indole ring system, is synthesized via a complex and energetically demanding pathway, represents a precursor of vitamin B\(_{3}\) (nicotinic acid), and belongs to the least abundant amino acids in proteins.

The target genes for determining transcriptional response thresholds for each associated carbohydrate or amino acid in *P. inhibens* DSM 17395 were selected based on known substrate-specific profiles of the encoded proteins [Drüppel et al., 2014; Wiegmann et al., 2014]. Figure 1 maps the selected genes to their respective positions in carbohydrate uptake (Fig. 1a), degradation pathway (Fig. 1b) and genomic context (Fig. 1c). The selected “degradation” genes were: *nagK*, encoding *N*-acetylglucosamine kinase (catalyzes 1\(^{st}\) step in *N*-acetylglucosamine degradation); *mttK*, encoding mannitol 2-dehydrogenase (catalyzes 1\(^{st}\) step in mannitol degradation); *xylA*, xylose isomerase (catalyzes 1\(^{st}\) step in xylose degradation); *mccB*, encoding β-subunit of methylcrotonyl-CoA carboxylase (catalyzes 4\(^{th}\) step in leucine degradation); *paaA*, encoding α-subunit of ring 1,2-phenylacetyl-CoA epoxidase (catalyzes 3\(^{rd}\) step in phenylalanine degradation); and *kynA*, encoding tryptophan 2,3-dioxygenase (catalyzes 1\(^{st}\) step in tryptophan degradation). The selected “uptake” genes encode solute-binding proteins of ABC-transporters for the three tested carbohydrates: *c27930*, *N*-acetylglucosamine-specific; *c13210*, mannitol-specific; and *xylF*, xylose-specific. However, the proteogenomic datasets available from previous studies together with current bioinformatical analyses did not allow unambiguous assignment of specific transporters (systems) for the uptake of the three tested amino acids.

Experimental Design

The setup of experiments followed the same design as previously conceived for betaproteobacterial *Aromatoleum aromaticum* EbN1\(^{T}\), where transcriptional response thresholds for aromatic compounds were determined [Vagts et al., 2020, 2021]. In the present study, succinate-adapted cells of *P. inhibens* DSM 17395 were grown with a limiting supply of succinate (8 mM), upon complete depletion of which a single pulse of an individual carbohydrate or amino acid was given to each culture, resulting in starting concentrations extending from 100 µM down to 10 nM. Reproducibility of growth curves and sampling time points in relation to the effector pulse (−5, 5, 15, 30, 60, 120, and 180 min) is exemplified for *N*-acetylglucosamine in Figure 2 (for the other tested effectors, see online suppl. Fig. S1–S5; for all online suppl. material, see www.karger.com/doi/10.1159/000524702). Differential expression of targeted “degradation” and “uptake” genes was related to the individual expression levels before the effector pulse (−5 min sample).

In vivo Response Thresholds of “Degradation” Gene Transcription

Irrespective of the administered effector substrate and its concentration, no obvious growth was observed after depletion of the primary growth substrate succinate, but rather the optical density (OD) values declined. This correlates with the increased formation of rosette-like aggregates typical of *P. inhibens* DSM 17395 [Drüppel et al., 2014; Segev et al., 2015].

The time-resolved transcript profiles for the three tested carbohydrates were overall similar with transcript abundance decreasing even more rapidly with lower pulse concentrations (Fig. 3; online suppl. Table S1). However, two distinct differences were also observed.
Fig. 2. Cultivation of *P. inhibens* DSM 17395 for targeted transcript analysis in response to N-acetylglucosamine. Upon depletion of the primary growth substrate succinate after ~9 h, N-acetylglucosamine was added (red dashed line) yielding a distinct concentration as indicated in the zoom-in boxes. In each case, triplicate cultures were performed (note error bars, standard deviation). The grey dashed lines indicate the sampling time points for transcript analysis. Analogous growth experiments for the other tested compounds are illustrated in the online supplementary material (mannitol, online suppl. Fig. S1; xylose, online suppl. Fig. S2; leucine, online suppl. Fig. S3; phenylalanine, online suppl. Fig. S4; and tryptophan, online suppl. Fig. S5).
While in case of N-acetylglucosamine (Fig. 3a) and xylose (Fig. 3c), strong transcriptional responses of the targeted nagK and xylA genes were observed already 5 min past effector pulse, this only occurred after 15 min with the mtlK gene for mannitol (Fig. 3b). Furthermore, the response thresholds differed in the following decreasing order: 50–100 nM for N-acetylglucosamine, 10–50 nM for xylose, and around 10 nM for mannitol. In case of the three tested amino acids, the transcript profiles were less congruent. For leucine, a response threshold could not be determined unambiguously since mccB transcript formation was observed at all administered concentrations across all sampling time points, albeit at 10–100 µM with highest abundances (Fig. 3d). This is most probably due to the presence of leucine even in the exometabolome of controls receiving substrate-free mock pulses (see below).
paragraph). The transcriptional response thresholds of \textit{paaA} and \textit{kynA} for phenylalanine and tryptophan, respectively, were found to lie between 100 and 50 nM (Fig. 3e, f).

The conspicuous tailing of the \textit{mccB} transcripts across the investigated concentration and time matrix – not observed with the tested carbohydrates – prompted the following hypothesis. Leucine as an abundant protein con-
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A constituent may accumulate extracellularly to an extent large enough to induce \( \textit{mccB} \) expression even though exogenous leucine added as an effector should have long been consumed. Such an intrinsic leucine accumulation could result from proteolytic activities during lysis or leakage of stationary phase cells with extracellular leucine accumulating faster than being taken up. To check this assumption, a targeted metabolite analysis was conducted differentiating between intra- and extracellular space of \( \textit{P. inhibens} \) DSM 17395 (Fig. 4). Among the tested carbohydrates, only \( N \)-acetylglucosamine was detected intracellularly and none of them in the cell-free culture supernatant. Most of the tested amino acids were detected at significant levels in the cells, but only leucine and structurally related isoleucine and valine were detected in the cell-free culture supernatant. Thus, intrinsic accumulation of leucine represents a likely explanation for the observed \( \textit{mccB} \) transcript profiles in the absence of exogenous leucine. Noteworthy, also \( \textit{paaA} \) (phenylalanine) and \( \textit{kynA} \) (tryptophan) show a low background expression level (for comparison, see online suppl. Fig. S6), which could result from an analogous intrinsic occurrence of phenylalanine and tryptophan, albeit at lower concentrations than leucine.

\textbf{In vivo Response Thresholds of “Carbohydrate Uptake” Gene Transcription}

The effector concentration-dependent, time-resolved transcript profiles for the selected carbohydrate-binding proteins of ABC-transporters are shown in Figure 5 (online suppl. Table S2). Notably, the general profiles are in accord with those described above for the selected “degradation” genes (Fig. 3a–c) including to a certain extent also the determined transcriptional response threshold: 0.1–1 µM for \( c27930 \) (\( N \)-acetylglucosamine), below 10 nM for \( c13210 \) (mannitol) and \( xylF \) (xylose).

\textbf{Theoretical Considerations on Effector Equilibria}

A previous study with \( \textit{A. aromaticum} \) EbN1\(^T\) determined in vivo transcriptional response thresholds toward the alkylphenols \( p \)-cresol and \( p \)-ethylphenol (effectors) in the range of 1–10 nM. Associated theoretical considerations revealed that the effector equilibrium is attained by passive diffusion within milliseconds, yielding merely 20 solute molecules inside the cytoplasm at a 10 nM solute concentration in the extracellular space (see Fig. 5 in [Vagts et al., 2020]). Subsequently, in vivo response thresholds of \( \textit{A. aromaticum} \) EbN1\(^T\) toward lignin-derived phenylpropanoids were found to cover a similar range (1–50 nM). From comparison with reported dissociation constants and abundances of periplasmic solute binding proteins (SBPs), it was concluded that uptake of these lipophilic compounds via ABC-transporters should not play a role in attainment of the effector equilibria at...
10 nM solute concentration, which rather proceeds by passive diffusion, too (see Fig. 8 in Vagts et al. [2021]).

Compared to the aforementioned in vivo response thresholds of *A. aromaticum* EbN1T, those determined here for *P. inhibens* DSM 17395 fall in the same range, albeit shifted to somewhat higher nanomolar concentrations. The higher threshold toward N-acetylg glucosamine (50–100 nM) most likely results from its intracellular presence as a building block of peptidoglycan, agreeing with the highest threshold of 10–100 nM for the associated SBP. It would be inefficient for the cell to synthesize the SBP for N-acetylg glucosamine at zero to very low ambient concentrations because the SBP would then bind endogenically produced N-acetylg glucosamine and transport it back into the cell via the ABC-transporter rather than incorporate it into the peptidoglycan layer. We surmise that the periplasmic concentration of free N-acetylg glucosamine under non-inducing conditions does not greatly exceed 10 nM.

As gram-negative bacteria, both *A. aromaticum* EbN1T and *P. inhibens* DSM 17395 have similar cell envelope architecture, which implies similar transport properties for passive diffusion of solutes into and out of the cell. Therefore, the general considerations derived for the gram-negative cell envelope of *A. aromaticum* EbN1T [Vagts et al., 2020] also apply to the passive uptake of solutes in *P. inhibens* DSM 17395. With this in mind, the tested carbohydrates are up to 5 orders of magnitude less lipophilic and thereby less membrane-permeable than the aromatic compounds studied with *A. aromaticum* EbN1T, e.g., XlogP3 \text{mannitol} = −3.1 versus XlogP3 \text{p-cresol} = +1.9 (xylose and N-acetylg glucosamine are 4- and 25-times less lipophilic than mannitol). This markedly lower degree of lipo philia implies passive diffusion time scales of the order of 10 s–100 s of seconds (rather than milliseconds as with p-cresol [Vagts et al., 2020]), which severely limits the efficiency of substrate uptake by passive diffusion. In contrast, the genes encoding the SBPs of mannitol and xylose have nanomolar response thresholds (Fig. 5b, c), which suggests that uptake via high-affinity ABC-transporters has an important role in attaining the respective transmembrane equilibria.

The observed delay of *mtlK* expression (15 min after pulse) was consistent across all tested mannitol concentrations and contrasted with the expression profiles of the other five “degradation” and three “carbohydrate uptake” genes (5 min after pulse). Since the membrane-permeability properties of the three carbohydrates are fairly comparable, they should not be the underlying cause of the delay. Also, a longer retention time of mannitol at its SBP compared to the other two carbohydrates appears unlikely. Furthermore, no alternative reaction sequence (e.g., involving a mannitol phosphotransferase and consecutive mannitol-1-phosphate 5-dehydrogenase forming fructose-6-phosphate) for channeling mannitol into the ED-pathway can be inferred from genome predictions, which could explain the delay if reacting faster. Thus, the observed delay of *mtlK* expression remains elusive at present, including unknown promoter efficiency.

**Implications for Persistence of DOM in Marine Systems**

The waterbodies of the world’s oceans contain a total \~662 Pg carbon as DOM, representing the 200-fold equivalent of total carbon in marine biomass and yielding an average concentration of 34 – \~85 \text{µmol kg}⁻¹ [Hansell et al., 2009]. DOM is presumably composed of millions of different compound structures, resulting in (below) picomolar concentrations each [Hansell and Orellana, 2021]. The composition of DOM is dynamically altered via the metabolism of heterotrophic microorganisms, depending on the bioavailability and reactivity of its individual components. In this context, DOM is divided into labile (readily biodegradable) and recalcitrant (resistant to biodegradation; average half-life time of several thousand years) fractions [Kujawinski, 2011; Hansell, 2013; Benner and Amon, 2015]. The persistence of DOM was long attributed to intrinsic molecular properties (“intrinsic recalcitrance” paradigm) of the respective components hampering their biodegradation. By contrast, the more recent “emergent recalcitrance” concept assumes that the molecule-microbe-interactions on an ecosystem level govern the transitions between lability and recalcitrance [Mentges et al., 2020]. Labile DOM can be metabolized within hours and reintegrated into the ambient DOM pool, which thereby becomes enriched in carbon [Hach et al., 2020].

Cultures of *P. inhibens* DSM 17395 grown with a single carbon source were shown to produce a highly diverse exometabolome composed of 21,105 molecular masses [Noriega-Ortega et al., 2019]. The findings of the present study with *P. inhibens* DSM 17395 may contribute two perspectives to the “emergent recalcitrance” concept. First, the rapidness (within min) of detectable transcriptional response even to effector concentrations in the nanomolar range agrees well with the instantaneous re-mineralization of 13C-labelled DOM by microbial communities in oligotrophic Atlantic surface waters [Hach et al., 2020]. Second, the determined thresholds of transcriptional responsiveness (<10–100 nM) of “degrada-
tion" and "uptake" genes are orders of magnitudes higher than the assumed below picomolar concentrations of individual DOM components in the marine system. Nevertheless, the response thresholds agree well with a previous study showing monosaccharides to have average concentrations of 4.3 μM in various ocean waters [Pakulski and Benner, 1994]. To reconcile this apparent discrepancy between pico- and nanomolar concentrations, analytic approaches need to comprehensively resolve structures and quantities of DOM components in oceanic provinces differing in nutrient availability. One may speculate that the observed thresholds reflect the potential of the marine heterotrophic *P. inhibens* DSM 17395 to rapidly respond to sudden increases in nutrient supply. Such “feast” scenarios are regularly encountered, e.g., in the microenvironment surrounding phytoplankton cells [Mitchell et al., 1985; Smirga et al., 2016], during the collapse of seasonal algal blooms [Teeling et al., 2012; Kuhlisch et al., 2021] or in nutrient- and species-rich marine upwelling ecosystems [Fréon et al., 2009]. Below the determined response thresholds of *P. inhibens* DSM 17395 (“famine” scenario in oligotrophic systems), growth- or maintenance-supporting DOM components may simply escape biodegradation due to non-expression of genes related to their uptake and degradation. However, a previous study with *A. aromaticum* EbN1[T, anaerobically cultivated in chemostats with benzoate as sole source of carbon and energy, revealed at μmin, a widely diversified formation of proteins for the uptake and degradation of various aromatic and aliphatic substrates despite their absence in the provided cultivation medium. This observation was interpreted as a prospective survival strategy (“preparedness for future nutritional opportunities”) enabling cells to instantaneously utilize substrates the moment they become available [Trautwein et al., 2012]. Thus, heterotrophic pelagic bacteria might be able – via presently unclear global regulatory mechanisms (e.g., derepression) operating under extreme substrate limitations – to metabolize substrates evading specific sensory recognition at subnanomolar ambient concentrations.

**Materials and Methods**

**Bacterial Strain, Medium, and General Cultivation Conditions**

*P. inhibens* DSM 17395 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). Routine cultivation, substrate-adaptation, and main growth experiments were performed in 250 mL defined marine mineral medium [Zech et al., 2009] containing 8 mM succinate (1,000-mL Erlenmeyer-flasks, 28°C, 100 rpm). Prior to any experiments, *P. inhibens* DSM 17395 was first revived by inoculating a dilution series (10⁻¹ to 10⁻⁶) with a glycerol stock and adapting the cultures to the aforementioned conditions over five passages. Main cultures were then inoculated from actively growing pre-cultures (½ ODmax). Organic substrates were provided from sterile stock solutions. Purity of the cultures was confirmed by routine microscopic examination (Axioskop; Zeiss AG, Göttingen, Germany) and by streaking cells on MB agar plates at the end of each growth experiment. All chemicals used were of analytical grade.

**Monitoring Growth and Quantification of Growth Substrate**

OD was determined at 600 nm using a UVmini-1240 spectrophotometer (Shimadzu, Duisburg, Germany). The depletion of succinate during the growth of *P. inhibens* DSM 17395 was determined by HPLC, as recently reported by Vagts et al. [2021]. An Ultimate 3000 system (ThermoFisher, Germering, Bavaria, Germany) was used, which was equipped with an RI-detector (Shodex RI-101; Showa Denko GmbH, München, Germany) and operated at 75°C. Separation was achieved on an Eurokat H column (300 x 8 mm, 10-μm bead size; Knauer, Berlin, Germany), using 5 mM H2SO4 as eluent administered at 1.2 mL min⁻¹. The retention time was 5.6 min and the dynamic range was from 25 μM to 10 mM.

**Growth Experiments for Determination of Response Thresholds**

The transcriptional responsiveness of *P. inhibens* DSM 17395 was determined with a concentration series of N-acetylglucosamine, mannitol, xylose, leucine, phenylalanine, and tryptophan, respectively, applying an experimental setup essentially as described in previous studies on *Aromatoleum aromaticum* EbN1[T [Vagts et al., 2018, 2020, 2021]. In short, upon complete depletion of the growth substrate succinate, cells of *P. inhibens* DSM 17395 were exposed to a single pulse of each tested carbohydrate and amino acid, resulting in extracellular concentrations ranging from 100 μM down to 10 nM. Subsequently, samples for continued monitoring of growth and for time-resolved transcript profiling were retrieved from the cultures as described previously [Vagts et al., 2020]: 5 min prior as well as 5, 15, 30, 60, 120, and 180 min after the addition of either carbohydrate or amino acid (effector).

**Cell Harvesting for Transcript Analyses**

Retrieval and processing of samples was conducted as previously described [Vagts et al., 2020]. Essentially, RNAprotect Bacterial Reagent (Qiagen, Hilden, Germany) was added to sampled culture broth (5 mL). After mixing, the suspension was incubated for 5 min at room temperature and centrifuged (4,800 g, 10 min, 4°C). Pellets were washed with 0.5 mL RNAprotect Bacterial Reagent (Qiagen), anew centrifuged, shock frozen in liquid N2, and stored at −80°C until further analyses.

**Cell Harvesting for Metabolite Analyses**

The metabolite analyses were based on 4 biological replicates per investigated time point and involved nine parallel cultures to provide sufficient sample material. The time points were ½ OD_max, ⅛ OD_max as well as 5 min prior to and 5, 15, 30, 60, 120, and 180 min after addition of a mock pulse (sterile medium without effector). In order to organize the loss of culture broth (50 mL per sample) as evenly as possible across the nine parallel cultures, the individual samples were retrieved from groups of four parallel cultures in a continuous order. The samples were immediately put on ice and centrifuged (12,000 g, 10 min, 4°C). A 2 mL subsample of each supernatant was transferred to a 2-mL micro reaction tube, shock-
frozen in liquid N2 and stored at −80°C until further analyses of the extracellular metabolome (exometabolome). The remaining supernatant was discarded, the pellet resuspended in 1.5 mL sterile mineral medium and transferred into a pre-weighted 2-mL micro reaction tube. After a new centrifugation (12,000 g, 10 min, 4°C), the resultant pellet was shock-frozen in liquid N2 and stored at −80°C until further analyses of the intracellular metabolome.

Preparation of Total RNA
Preparation of total RNA was conducted according to standard protocols [Oelmüller et al., 1990; Lahme et al., 2014] and as previously described [Vagts et al., 2020], involving the following main steps. Cell pellets were treated twice with hot acidic phenol (Roti® Aqua-Phenol; CarlRoth, Karlsruhe, Germany). After transfer of the resultant aqueous phase into a 2-mL 5PRIME phase lock gel tube (Quantabio, Beverly, MA, USA), one volume of phenol:chloroform:isoamylalcohol (25:24:1) was added. The tube was then gently inverted for 5 min and centrifuged to precipitate the nucleic acids, which were washed with ice-cold ethanol, dried, and resuspended in RNase-free water. Digestion with DNase I (RNase-free; ThermoFischer) was verified via PCR targeting the nagK gene and using genomic DNA of P. inhibens DSM 17395 as a positive control. RNA quality of all 903 samples underlying the transcript profiles was controlled using an Experion automated electrophoresis station (Bio-Rad, Hercules, CA, USA), confirming the integrity of rRNAs as well as the ratio between 23S and 16S rRNA. RNA concentrations were determined using a microplate reader (SPECKTROstar® Nano; BMG Labtech, Ortenberg, Germany). RNA samples were stored at −80°C until further analysis. All chemicals used for RNA preparation were of molecular biology grade.

Transcript Profiling by qRT-PCR
Profiling of transcript abundances by means of quantitative reverse transcription PCR (qRT-PCR) was performed as previously described [Vagts et al., 2018]. Primers specifically targeting the selected “degradation” and “uptake” genes were designed using the Primer3 software package (version 4.1.0; ) and are compiled in Table 1. 150 ng of total RNA were used for cDNA synthesis and real-time PCR. Samples were prepared using the Brilliant III ultrafast SYBR green qRT-PCR master mix (Agilent, Santa Clara, CA, USA)

### Table 1. Oligonucleotide primers used for targeted transcript profiling

| Primera | Target gene | Nucleotide sequence (5’→3’) | Product length, bp | PCR efficiencyb |
|---------|-------------|----------------------------|--------------------|-----------------|
| **Degradation** | | | | |
| N-acetylglucosamine | | | | |
| nagK_582_F | nagK | GATTGTCCGTTTCGCCAGAGTAATCCGCCCACATCCGCCAG | 234 | 1.98 |
| nagK_815_R | | | | |
| Mannitol | mtlK | GTGTGGCTTATGACGGCTCA CGGCTGCTCTGATAGATCCC | 269 | 1.96 |
| Xylose | xylA | AAAGCCACAAAGCCTGAAC ACAGATCGGTGTTGAGGAG | 267 | 2.06 |
| Phenylalanine | paaA | CGGCATCATGAACCAGGT ACATCATCGCGCAGGATACC | 201 | 2.01 |
| Leucine | mccB | TTACCTGGCCGAGATGACG AGTGACAAGATTTGCAGCA | 250 | 1.98 |
| Tryptophan | kynA | AAGTCTACCCGCAACACCGAG TCCAGCAGTCCGTTTCAGGT | 182 | 2.05 |
| **Uptake** | | | | |
| N-acetylglucosamine | c27930 | AACATCTTCACTCTGGCCCG TCCTATTGCCACCTCATG | 299 | 2.16 |
| Mannitol | c13210_301_F | CTAAACGATCTGCCTGCCGA TTGGACATGGCGGTAGGAA | 296 | 2.13 |
| Xylose | xylF | CGGTGTTAGCCGAGATGATCA ATACAGTCTGGGCTCCCCAGTG | 287 | 2.21 |

a F, forward primer; R, reverse primer. b Mean value of all performed qRT-PCR experiments.
Responsiveness of *P. inhibens* and analyzed with a CFX96 real-time system (Bio-Rad) via 40 cycles, using the following setting: 3 min at 95°C (initiation); 10 s at 95°C (denaturation); 2 times 30 s plus 5 s at 60°C (annealing, extension, and real-time detection). Melting-curve analysis (60–90°C in steps of 0.5°C) was used to verify the specificity of accumulated products. Samples taken 5 min prior to addition of either carbohydrate or amino acid were used as reference and all samples retrieved thereafter represented the test states. Per time point, 3 biological replicates were analyzed each with 3 technical replicates. All samples related to one effector concentration were analyzed in a single batch (on the same multwell plate). Differences in relative transcript abundance were calculated and primer-specific PCR-efficiencies (*E*) determined as previously described [Ramakers et al., 2003; Vagts et al., 2018].

**Identification and Quantification of Intra- and Extracellular Carbohydrates and Amino Acids**

Metabolome extraction and analysis were performed as described previously [Will et al., 2019]. Briefly, the biomass was re-suspended in methanol (30 µL per 10 mg wet weight spiked with 4% ribitol stock solution (0.2 g L⁻¹ in water) as internal standard) followed by cell lysis in an ultrasonic bath for 10 min at room temperature. The same volume of water was added followed by vigorous shaking for 1 min. Subsequently, 500 µL of chloroform were added and vigorously shaken for 1 min followed by centrifugation (12,000 g, 5 min). Fifty microliters of the polar phase were transferred into vials and dried in a vacuum concentrator for 1 h. For determination of extracellular metabolites, 50 µl of the supernatant were spiked with 20 µL of 4% ribitol stock solution (0.2 g L⁻¹ in water) in methanol and dried for 1 h. The external calibration curve was prepared as the supernatant using authentic standards (Sigma-Aldrich/Merck, Darmstadt, Germany) using carbon source-free medium for dilution. Metabolome analysis was performed on an Agilent GC-MSD system (7890B coupled to a 5977 GC) equipped with a high-efficiency source (Agilent Technologies, Waldbronn, Germany). A two-step derivatization was performed with a methoxyamine hydrochloride solution (20 mg mL⁻¹ in pyridine) and *N*-methyl-*N*-(trimethylsilyl)-trifluoracetamide. One microliter was injected into a multimode inlet in pulsed splitless mode and separation was conducted on an Agilent VF-5ms column with a helium flow of 1.2 mL min⁻¹. The oven temperature was hold at 70°C for 6 min and then linearly increased with 6°C min⁻¹ up to 325°C. Ions were detected in scan mode from 70 to 700 m/z with 2.3 scans s⁻¹. Data analysis was performed as described previously [Neumann-Schaal et al., 2015; Hofmann et al., 2018]. The detection limits (nmol mg⁻¹) were as follows: 0.001 for leucine, isoleucine, valine, phenylalanine, mannotol and sucrose; 0.002 for methionine and tryptophan; 0.003 for xylose; 0.004 for lysine and threonine; 0.011 for *N*-acetylglucosamine; and 0.290 for histidine.

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**Statement of Ethics**

Ethic approval was not required.

**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

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**Author Contributions**

Ralf Rabus conceived the study; Arne Weiten, Kristin Kalvelage, Ramona Buschen, and Sabine Scheve conducted the cultivation experiments; Sabine Scheve conducted HPLC analyses; Arne Weiten, Kristin Kalvelage, and Sabine Scheve performed the transcript analyses, Meina Neumann-Schaal conducted the metabolite analyses; Michael Winklhofer did mathematical calculations; Ralf Rabus wrote the manuscript with contributions from Arne Weiten, Kristin Kalvelage, Meina Neumann-Schaal, and Michael Winklhofer. All the authors have agreed to the final version of the manuscript.

**Data Availability Statement**

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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