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# Aerobic Degradation of the Antidiabetic Drug Metformin by Aminobacter sp. Strain NyZ550

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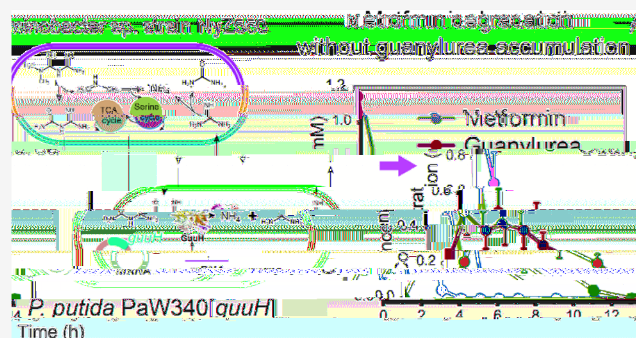
ABSTRACT: Metformin is becoming one of the most common emerging contaminants in surface and wastewater. Its biodegradation generally leads to the accumulation of guanylurea in the environment, but the microorganisms and mechanisms involved in this process remain elusive. Here, Aminobacter sp. strain NyZ550 was isolated and characteri ed for its ability to grow on metformin as a sole source of carbon, nitrogen, and energy under o!ic conditions. "his isolate also assimilated a variety of nitrogenous compounds, including dimethylamine. Hydrolysis of metformin by strain NyZ550 was accompanied by a stoichiometric accumulation of guanylurea as a dead-end product. #ased on ion chromatography, gas chromatography−mass spectrometry, and comparative transcriptomic analyses, dimethylamine was identi$ed as an additional hydrolytic product supporting the growth of the strain. Notably, a microbial mi!ture consisting of strain NyZ550 and an engineered Pseudomonas putida %a&'(0 e!pressing a guanylurea hydrolase was constructed for complete elimination of metformin and its persistent product guanylurea. ) verall, our results not only provide new insights into the metformin biodegradation pathway, leading to the commonly observed accumulation of guanylurea in the environment, but also open doors for the complete degradation of the new pollutant metformin.




KEYWORDS: bacteria, biodegradation, emerging contaminant, guanylurea, metformin

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## #. $NTR D%CT$ N

%harmaceuticals in the environment are considered emerging contaminants and are of global concern due to their gradually revealed ris*s to ecosystems and human health.[+] %harmaceutical pollution is primarily due to the use of pharmaceuticals by humans or animals, resulting in their continuous release into the environment through wastewater treatment plants ,&&"%s- worldwide.' Most pharmaceuticals are synthetic compounds or natural products with modi$cations, a/ecting biological targets even at e!tremely low concentrations. "heir presence in the environment has adverse e/ects on the ecosystems, a0uatic organisms, and humans.[',(] However, these e/ects are largely underestimated due to the lac* of comprehensive data on their unpredictable synergistic to!icity and the ris*s of their structurally diverse products, as well as on the biodegradability[5] and environmental fate of pharmaceuticals.[+]

Metformin ,M1"- ,+,.-dimethylbiguanide- is a synthetic guanidine derivative used as a blood glucose-lowering drug for type II diabetes. 2urrently, more than 500 million people worldwide have diabetes, primarily type II diabetes, with the number estimated to increase to 34' million by .0(5.[5] 6ue to the worldwide prevalence of diabetes and the need for long-term medication, M1" has become one of the most commonly used prescription drugs.[3] However, M1" is not metaboli ed by the human body and is e!creted as a prototype in urine and feces after oral administration.[4,7] "herefore, most M1" will converge on &&"%s, leading to high concentrations of M1" in the wastewater of &&"%s, with concentrations up to 30. μg 8[−+] observed in 9nited : tates.[+0] 6uring ; ow through the &&"%s, M1" is mainly transformed by biological processes. "he present treatment of &&"%s shows incomplete elimination of M1" with removal rates ranging from (+< to more than 74<, and ultimately signi$cant amount of M1" from &&"%s is released into the environment.[++] 2onse0uently, the worldwide occurrence of M1" has been observed in surface, ground, drin*ing and coastal waters, sludges, and soils.[',+0,+.,+'] It has recently been categori ed as one of the top pharmaceuticals present in the environment.[',+(] M1" e!posure e!hibits adverse e/ects on a0uatic life[+5−+3] and can accumulate in plants.[+4] In addition, its transformation

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products such as guanylurea ,>9?-[+7,.0] and chlorination byproducts,[.+] appear to be to!ic and widely distributed.[+.,..−.5]

"he widespread occurrence and obvious ris*s of M1" and its derivatives necessitate remediation of M1"-contaminated environments. 6eveloping biodegradation technologies compatible with current wastewater treatment processes is considered a cost-e/ective and promising strategy. However, this is limited by a lac* of available M1"-degrading bacterial strains and *nowledge of their degradative properties. Microbial consortia from soils or activated sewage sludge have been demonstrated to be able to degrade M1" and >9? under aerobic or anaerobic conditions.[+7,.0,.3−'+] It has been suggested that aerobic conditions are preferred for M1" degradation, whereas >9? degradation is faster under anaerobic conditions.[.7] However, aerobic degradation of >9? was promoted by the addition of glucose as a cosubstrate, suggesting that >9? can be readily used as a sole nitrogen source.['0] Pseudomonas mendocina >9 was recently isolated from &&"%s for its ability to grow on >9? as a sole nitrogen source.['.] ? novel hydrolase was identi$ed that cataly ed the initial hydrolysis of >9?, and the resulting ammonium and guanidine were assimilated by this strain. "ill date, only a single bacterial strain ,Aminobacter sp.- capable of using M1" as the sole carbon source has been isolated from activated sludge, and the strain converts M1" into the dead-end product >9?, but further details of this degradation have not been investigated.['+]

In this study, we determined the concentrations of M1" and >9? in wastewater from +. &&"%s in 2hina and the M1"-degrading capacity of microbial consortia from these samples. "he results helped us successfully isolate a M1" utili er, the Aminobacter sp. strain NyZ550, capable of growing on M1" as a sole source of carbon, nitrogen, and energy. ?n investigation of the degradation of M1" by strain NyZ550 revealed the following characteristics@ ,i- strain NyZ550 has a robust ability to degrade M1", ,ii- strain NyZ550 is a versatile metaboli er of nitrogen-containing compounds, and ,iii- the degradation of M1" produces >9? and dimethylamine ,6M?- via hydrolysis, with the former a dead-end product and the latter supporting the growth of strain NyZ550. 2onse0uently, a microbial mi!ture composed of strain NyZ550 and an engineered Pseudomonas putida %a&'(0 e!pressing >9? hydrolase e!hibits a capacity for both M1" and >9? degradation.

## &. MAT'R$A(S AND M'T) DS

&.#. **Reagents.** M1" ,+,+-dimethylbiguanide hydrochloride-, guanylurea sulfate salt hydrate, and dicyandiamide were purchased from "2I ,:hanghai, 2hina-. Methylamine, 6M? ,(0< in water-, trimethylamine ,'0< in water-, and water-[+4]) were purchased from :igma-?ldrich ,:t. 8ouis, 9:?-. >uanidine and +,+-dimethylguanidine were purchased from AuanAe #io-"echnology ,:hanghai, 2hina-. #iuret, +-methylbiguanide, and biguanide were purchased from #idepharm ,:hanghai, 2hina-. ?ll chemicals had a purity of B73<. ) ther commercial inorganic chemicals of analytical grade were used in this study. 9ltrapure water and H%82 grade methanol were used to prepare the samples and mobile phases for ultra-performance li0uid chromatography ,9%82-.

&.&. ***aste+ater Samp!e Co!!ection.** &astewater samples were collected from domestic &&"%s from ++ cities in 2hina in ?pril .0.+. 1ach &&"%s had a treatment capacity of 45,000−.00,000 m' per day, and all of them had an aeration tan* where the wastewater was sampled. #iodegradability tests were conducted within + wee* after the samples were transported to the laboratory. Cor 82−M: analyses, +0 m8 of each sample was pretreated by direct $ltration with 0.. μm nylon $lters. Ciltered samples were ad0usted to pH 3.0 with + M Na) H or H2I and stored at −.0 °2.

&.,. **-atch Cu!tures.** #atch e!periments to test the biodegradability of the wastewater samples toward M1" were performed in .50 m8 1rlenmeyer ;as*s sealed with the polytetra;uoroethylene ventilatory $lm ,pore diameter 0..− 0.' μm-. "he ;as*s containing +00 m8 of minimal salt medium ,M:M, pH 3.0-[''] and +.5 mM M1" were added with an appropriate volume of the wastewater sample as inoculum to a density of appro!. +0( to +0[5] colony forming units ,2C9s- per m8, determined by serial dilution of wastewater and plate count of microorganisms grown on the "ryptic :oy #roth agar plate. "he cultures were incubated at '0 °2 in a sha*ing incubator at +40 rpm. :amples were ta*en at appropriate intervals in triplicate for measuring the concentrations of M1" in the cultures. "he cultures were transferred to new ;as*s after +. days of incubation. ?n e0ual volume of autoclaved wastewater was inoculated as a control.

&... **$so!ation and /ro+th of M'T0%ti!i1ing Micro0organisms.** "he batch cultures capable of degrading M1" were used for further enrichment and isolation of M1" utili ers according to a method previously described.[''] "he pure isolates were identi$ed by amplifying and se0uencing the +5: rEN? gene se0uences using primers .3C ,5-?>?>"""->?"22">>2"2?>-' - and +(7.E ,5-"?2>?2"-"??2222??"2>2-' -. "he isolates were grown in M:M containing M1" or other substrates with indicated concentrations ,+−4 mM- at '0 °2. Medium was supplemented with 0..< ,wFv- ammonium sulfate as a nitrogen source or +0 mM glucose as a carbon source if necessary. "he population doubling time was estimated as a method previously described.['(] "he optimal growth conditions of the M1" utili er were tested by culturing the strain in M:M containing . mM of M1" under various pH, temperature, and salinity conditions. "he pH values were ad0usted by phosphoric acid or + M sodium hydro!ide.

&.5. **$sotope (abe!ing '2periment.** M1" was dissolved in H.[+4]) or H.) at a concentration of 0.+ M as the stoc* standard. "he M1" utili er NyZ550 was inoculated into +50 m8 of M:M containing . mM M1" and grown to the late e!ponential phase ,) $6_{500 nm}$ of appro!. 0.0(- at '0 °2. "hen, cells were harvested by centrifugation ,3000×g, +0 min, ( °2- and washed twice with 50 mM "ris−H2I bu/er ,pH 4.0-. "he cells were divided into two e0ual volumes, one of which was incubated with 500 μM M1" dissolved in H.[+4]) and the other with 500 μM M1" dissolved in H.). "he reaction mi!tures were incubated at '0 °2 for + h and the products were analy ed by 9%82-G")CM:. ?ll reactions were performed in triplicate.

&.3. **'n1yme Assays.** :train NyZ550 was grown on M1" and harvested as described above. "he pellet was suspended in M:M and then lysed by sonication. "he resulting cell lysate was centrifuged at +5,000×g for (0 min to remove debris, and the supernatant was used for en yme assays. "he reaction mi!tures ,0.5 m8- containing 500 μg of protein from the crude e!tract and 0.5−+ mM M1" in 50 mM "ris−H2I bu/er ,pH 4.0- were incubated at '0 °2. :amples were removed at appropriate intervals and analy ed by 9%82-G")CM:, gas chromatography−M: ,>2−M:-, and ion chromatography.



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" he spectrum change during the reaction was recorded using a 8ambda . 5 spectrophotometer ,%er*in1lmerF2etus, Norwal*, 2" -.

&.7. Ana!ytica! Methods. M1" and >9? were 0uanti$ed using an 9%82 system ,?gilent +. 50 In$nity II- connected in series with triple 0uadrupole mass spectrometry ," GM: FM: , ?gilent 5(30 system-. " he 9%82 system was e0uipped with an H#ridge phenyl column ,'.5 μm × +50 mm × ..+ mm-. Mobile phase ? was ultrapure water containing +0 mM ammonium formate ,adDusted to pH '.5 with formic acid-, and mobile phase # was methanol. " he 9%82 gradient started at 5< #, which was held for (.5 min, then increased to 75< at 4.5 min, where it remained until 7 min, and was then reduced to 5< at +5 min before a 5 min re-e0uilibration. " he binary mobile phase was set to a ; ow rate of 0.. or 0.( m8Fmin, and the inDection volume was +0 μ8. Cor M: analysis, electrospray ioni ation was used to generate two transition ions from analytes in the positive ioni ation mode, one transition ion for 0uanti$cation and the other for con$rmation. " he following ion transitions ,mFz- were used@ M1" +'0F50 and +'0F3', >9? +0'F50 and +0'F('. " he ioni ation parameters were set as follows@ the desolvation gas temperature was '.0 °2, the drying gas ; ow was 4 8Fmin, the nebuli er pressure was '5 psi, the capillary voltage was ( *I , and the collision energy was .0 eI . Guanti$cation was achieved using the e!ternal standard method and ?gilent MassHunter Guantitative ?nalysis software ,version #.04.00-. " he calibration curves were generated by calculating the pea* area versus the concentration of each target compound ," able : +-. " he limits of detection were +0 and +5 ngF8 for M1" and >9?, respectively.

" he ?gilent +. 70 In$nity II-?gilent 55(5 G" ) C M: system was used for 0ualitative analyses of >9? and other compounds, as indicated. " he 82 system was operated as described above, and G" ) C M: was performed using an electrospray source in the positive mode with a mass range of 50–500 mFz. " he ioni ation parameters were set as following@ the desolvation gas temperature was '00 °2, the drying gas ; ow was 5 8Fmin, the nebuli er pressure was (5 psi, and the capillary voltage was ( *I .

6M? was analy ed with an I2:-700 ion chromatograph ," hermo Cisher :cienti$c, 2?, 9:?- composed of a dual-piston pulse infusion pump system, eluent generator, and digital conductivity detector. " he separation was performed by a 6ione! Ion%ac 2+5 column ,5 × +50 mm- maintained at '0 °2 and a conductivity detector using an e!ternal calibration. " he eluent solution contained +0 mM methanesulfonic acid at a ; ow rate of + m8Fmin for 50 min. " he inDection volume was .5 μ8. In addition, 6M? was also identi$ed using a >2–M: G%. 0+0 :1 system ,:himad u 2orporation, =apan- in the :IM mode under the following conditions@ an :H-I-5M: column ,'0 m × 0..5 μm × 0..5 mm- was used and interface and source temperatures were ..0 °2. " he oven temperature procedure was 50 °2 for + min, increased to +50 °2 at +0 °2F min, held for + min, then increased to '00 °2 at .0 °2Fmin.

&.8. /enome and Transcriptome Se9uencing. " he M1" -grown NyZ550 cells were collected, as described above, and their genomic 6N? was e!tracted by an 9ltra2lean Microbial 6N? Isolation *it ,Mo#io 8aboratories, Inc., 9:?-. " he total 6N? of NyZ550 was se0uenced by an Illumina Hi:e0 platform with the paired-end mode. " he se0uenced reads were assembled using :) ?%denovo. software.[' 5] " he draft genome se0uence of strain NyZ550 was deposited in the N2#I database under the proDect %E=N?44. . . 4.

:train NyZ550 cells grown on glucose ,>89 group- or M1" ,M1" group- were collected and sent to the %ersonalbio " echnology 2o., 8td. ,:hanghai, 2hina- for EN? se0uencing. " otal EN? was e!tracted using a " EI ol-based method ,8ife " echnologies, 2?-, resulting in EN? samples with a concentration of more than .50 ngFμ8. " hen, EN? libraries of '00–(00 bp were constructed and se0uenced by the Illumina platform. " he adapter se0uences and low-0uality reads from the raw data were removed before gene e!pression analysis. " he gene e!pression levels were evaluated as fragments per *ilobase of transcript per million fragments ,C%JM-. " he fold change of gene e!pression was calculated by comparison of the C%JM values of the M1" group to those of the >89 group. " he di/erentially e!pressed genes ,61>s- were identi$ed by a |log. foldchange| B+ ,p-value K 0.05-. " he transcriptomic raw data were deposited to the N2#I database with accession numbers :EE. . 555734 and :EE. . 555733.

&.:. Degradation of M' T by a Microbia! Mi2ture. " he >9? hydrolase gene ,guuh, >en#an* accession number@ M#C4+5'00(.+- was synthesi ed by " sing*e #iotechnology ,:hanghai, 2hina-. " he guuh gene was ampli$ed by the primers guuh-: ,5 ->?2>>22?>" >??" " " 2?>>2" - >??>22?>2>-' - and guuh-? ,5 -" >?" " ?2>2- 2??>2" " 2>???>>" " " " >2?22?" " 2>?" >>-' - and then inserted into the pEJ(+5 vector by homologous recombination. " he guuh was e!pressed under the control of a tac promoter. " he construct pEJ(+5-guuh was then introduced into P. putida %a&'(0 by electrotransformation, as described previously.[' 5] :train %a&'(0 carrying pEJ(+5-guuh was grown in +50 m8 of 8# medium containing +0 μgF m8 tetracycline at '0 °2 to an optical density at 500 nm ,) $6_{500\ nm}$- of 0.'. Aminobacter sp. strain NyZ550 was grown in +50 m8 of M:M containing . mM M1" to the e!ponential phase. " he two cultures were harvested by centrifugation and washed twice with 50 mM " ris–H2I ,pH 4.0- and resuspended in M:M to a density of '.. × $+0^4$ cellsFm8. " he %a&'(0 cell suspension was divided into two parts, with one part was used for biotransformation of >9? and the other mi!ed with e0ual number of NyZ550 cells ,appro!. 5.( × $+0^7$ cells in total-, which was used as a microbial mi!ture for biotransformation of M1". " he biotransformation mi!tures were incubated in a sha*er at ..0 rpm, '0 °2. :amples were removed at appropriate intervals and analy ed using 9%82-M:FM:. 2ontrol e!periments were performed using %a&'(0 cells carrying the empty pEJ(+5 vector. ?ll the assays were tested in triplicate.

## ,. R' S%(TS AND D$SC%SS$ N

,.#. M' T and /%A * ere ; re4a!ent in Samp!es from * * T; s in China. " he concentrations of M1" and >9? in +. wastewater samples were measured by 9%82-" GM:FM:. ?s shown in " able +, M1" was detected in all samples with a large variation in concentrations, ranging from 0.+ to (45.5 μg 8⁻⁺. >9?, a *nown metabolite of M1", was detected in 7 of the +. samples with concentrations ranging from .0..5 to 545.5 μg 8⁻⁺. " he levels of M1" and >9? in each environmental sample e!hibited a negative correlation. In the nine samples with high >9? concentrations, the concentrations of M1" were merely in the range of 0.+–'+.4 μg 8⁻⁺, while the remaining three samples ,&H, N=., and :=Z- in which >9? was not detected had high M1" concentrations ,.(3..–(45.5 μg 8⁻⁺-. " his result is consistent with previous observations in samples from &&" %s[(] and is li*ely due to



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Table 1. Concent ations o! " ET and #$A in the Waste%ate o! " unicipal WWTPs ! o& 11 Cities in China[a]

| sites | concentrations ,µgF8-[b] | |
|---|---|---|
| | M1" | >9? |
| #ei!ing ,#=- | '+.33 ± 0.(+ | .55.05 ± 53.0+ |
| 2hang hou ,2Z- | 0.70 ± 0.05 | .0..5+ ± +(..( |
| >uang hou ,>Z- | 0.++ ± 0.0+ | (75.(+ ± +(5.44 |
| Hang hou ,HZ- | 0.+7 ± 0.0' | '44.53 ± +(4.54 |
| 8an hou ,8Z- | +.'. ± 0.'( | 547.57 ± ..7.05 |
| Nan!ing+ ,N=+- | 5.05 ± 0.(5 | '73.( ± 5+..7 |
| Nan!ing. ,N=.- | .(3.. ± (..4( | N6 |
| :hanghai ,:H- | '.'3 ± 0.5' | '3'.7' ± +..4' |
| :hi!ia huang ,:=Z- | '.0.5( ± +..+5 | N6 |
| &u!i ,&H- | (45.5. ± 33.53 | N6 |
| Hiamen ,HM- | +.+( ± 0.0' | 545.5 ± +'+.'4 |
| Auhang ,AH- | +..( ± 0.0( | .44.+( ± 73.'7 |

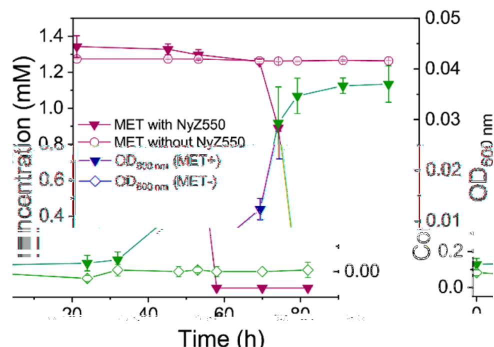
[a]N6@not detected. [b]"he data shown are average values of replicate samples ,n L '-.

the distinct ability of microorganisms to convert M1" into >9? in wastewater.

,.&. Distinct M'T0Degrading Capacity of $ndigenous Microorganisms from * aste+ater. "o compare the M1"-degrading abilities of indigenous microorganisms from di/erent wastewater samples, batch culture biodegradation tests were performed. M:M containing M1" ,from 0.7 to +.5 mM- was inoculated with wastewater from di/erent &&"%s, and M1" concentrations were measured over time ,Cigure +-. ?lmost complete degradation of M1" was observed in nine cultures within +0 days, whereas three cultures ,&H, N=., and :=Z- showed a poor capacity to degrade M1" within +. days. Interestingly, these three cultures were derived from the three original samples in which >9? was not detected but had high concentrations of M1" ,&H, N=., and :=Z, as listed in "able +-. "he +. cultures were further transferred after +. days to fresh media with +< ,vFv- inoculum and cultured under the same conditions. :imilar degradation behaviors were observed in the second transfers as in the $rst ones, e!cept for sample :=Z, which showed a complete degradation of M1" but over a longer period ,+. days-, indicating a possible microbial adaption or evolution for M1" degradation. 2ultures &H and N=. were still unable to degrade M1" even after e!tending the culturing period to .5 days. No evident elimination of M1" was observed in the controls of the autoclaved wastewater samples, indicating that M1" was not transformed through an abiotic manner under the conditions used.

,.,. M'T0%ti!i1ing -acteria * ere $so!ated from * * T; s. ?fter selective enrichment on M1" of the cultures e!hibiting M1" degrading capacity, $ve phylogenetically related Aminobacter sp. strains with the ability to grow on M1" were obtained from di/erent samples, and their +5: rEN? gene se0uences were deposited in >en#an* ,"able :.-. "hese strains e!hibited a similar degradation behavior for M1", and therefore, an isolate from sample :H ,designated Aminobacter sp strain NyZ550- was used for further analyses.

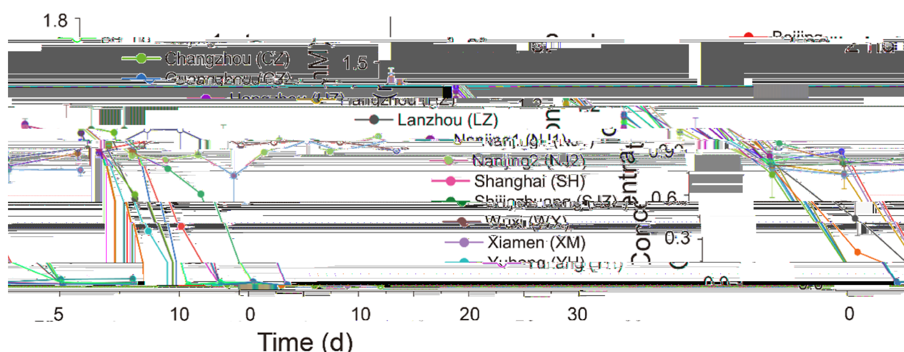
:train NyZ550 was shown to almost completely degrade ,77.4 ± 0.+(<- M1" within 50 h with an increase in cell biomass ,Cigure .-. No degradation of M1" was observed in




'i(u e ). >rowth of Aminobacter sp. strain NyZ550 on M1" ,M1"[M]- as a sole source of carbon, nitrogen, and energy. 2ontrols were conducted without inoculation or without addition of M1" ,M1"[−]-. >rowth ,green lines- is indicated by the increased optical density at 500 nm ,)6$_{500\ nm}$-, and the concentrations of M1" are shown in fuchsia lines.

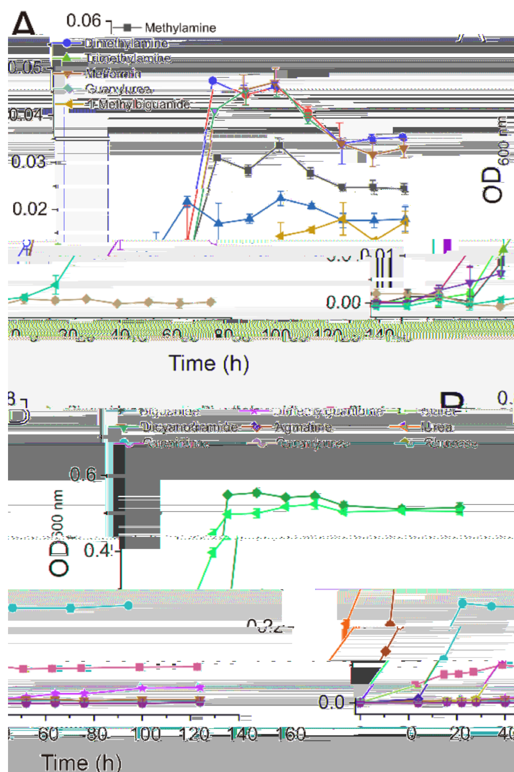
the control without inoculation of strain NyZ550, and no growth was observed in the control without the addition of M1" ,Cigure .-. "he results clearly established that the M1" loss was due to biodegradation by strain NyZ550. "he strain grew well at temperatures of +5−'0 °2, preferred neutral or al*aline conditions ,pH 5.0−+0.0-, and tolerated salinity up to .< ,as Na2I, wFv <- ,Cigure :+-.

,... Strain NyZ550 /ro+s on a <ariety of Nitro0genous Substrates. Nitrogen availability is often a growth-limiting factor for bacteria, and strains of Aminobacter are *nown for their versatile abilities to utili e nitrogenous compounds, thereby conferring them a selective advantage in the environment.['3,'4] Here, we tested the growth of strain NyZ550 on various nitrogenous compounds ,. mM- analogous to M1" or derived from M1". "he results showed




'i(u e 1. M1" degrading capacity of the microbial consortia from wastewater of +. &&"%s in 2hina. "he $rst transfer ,$rst- and second transfer ,second- were performed in minimal salt media containing M1".

that strain NyZ550 was able to use trimethylamine, 6M?, methylamine, and +-methylbiguanide as sole carbon, nitrogen, and energy sources for its growth ,Cigures '? and :.-. "he doubling time of strain NyZ550 grown on these substrates was +0.. ± +.', (.4 ± 0.., 5.5 ± +.5, and 4.5 ± +.5 h, respectively. "he low biomass yields could be due to the ineNciency in transportation, un-optimi ed catabolic ;u!, or to!icity caused by the substrates. ) ther nitrogenous compounds, including guanidine, biuret, urea, and agmatine, could only be used as the sole nitrogen source for growth by strain NyZ550 ,Cigure '#-. However, no clear growth was observed using biguanide, dicyandiamide, or the M1" degradation product >9? under the same culture conditions.

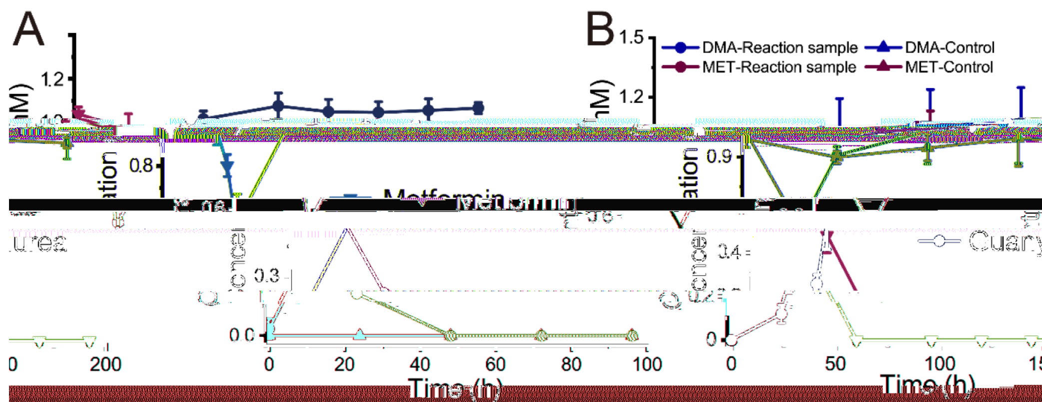



' i(u e *. >rowth of Aminobacter sp. strain NyZ550 on a variety of nitrogenous compounds. "he structures of these compounds can be found in Cigure :'. ,?- 2ompounds ,. mM each- indicated were used as the sole source of carbon and nitrogen. ,#- 2ompounds ,. mM each- indicated were used as the sole source of nitrogen, and +0 mM glucose was used as the carbon source.

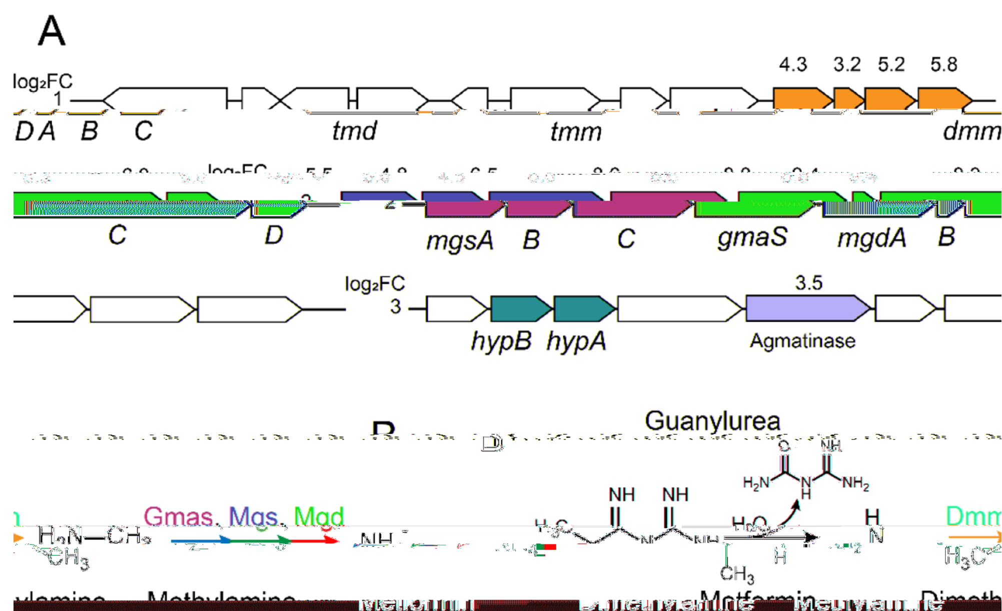
,.5. $denti=cation of the Catabo!ic ;roduct /%A. 2onsidering the e!tremely high proportion of >9? versus M1" in the sample :H, from which strain NyZ550 was isolated, it was assumed that >9? was a degradation metabolite. "herefore, identi$cation of metabolites produced during growth of strain NyZ550 on M1" was performed using 9%82-G") C M:. ? product pea* with a retention time of 4.7 min was con$rmed to be >9? based on comparison of the retention time and mass spectra ,0M M HPM@ +0'.05+5, mass accuracy@ +.7( parts per million 0ppmP error- with those of authentic >9? ,Cigure :(-. 6uring the growth of strain NyZ550, a stoichiometric accumulation of >9? was observed in the culture with the disappearance of M1", but >9? was not further degraded, even after culturing for an e!tended period after M1" was transformed ,Cigure (?-. Moreover, strain NyZ550 was unable to grow on >9? with or without the addition of glucose, indicating that >9? could not be used as the source of carbon or nitrogen for growth ,Cigure '-. "hese observations established that >9? was a dead-end product in M1" degradation by strain NyZ550. "his may well e!plain the accumulation of >9? in the wastewater ,sample :H- from which the strain NyZ550 was isolated. "his same catabolic process is li*ely to be present in other samples with high >9? concentrations in this study.

,.3. $ncorporation of the #8 Atom of )  into /%A during M' T Degradation. "o determine the source of the o!ygen atom incorporated into >9? and the reaction type for the formation of >9? from M1" degradation, biotransformation assay was performed to convert M1" into >9? by strain NyZ550 in H.+4), and the product was e!amined by mass spectrometry. "he molecular ion of +4) atom-labeled >9? ,mFz +05- appeared at a position two mass numbers higher than the corresponding ion of unlabeled >9? ,mFz +0'- ,Cigure :5-. "he observed product ratio of +4) labeled and unlabeled >9? was about .0@+, and this is li*ely because of the incorporation of H.) within the cells. "he result indicated that the +4) atom of H.+4) was incorporated into the >9?, thus clarifying that the reaction proceeded via hydrolysis.

,.7. $denti=cation of DMA as Another $nitia! Degradation ;roduct. "he evidence above dictates the biodegradation of M1" by NyZ550 with the accumulation of




' i(u e +. Identi$cation of the M1" degradation products >9? and 6M?. ,?- Guanti$cation of >9? formation along with the degradation of M1" during the growth of strain NyZ550. ,#- Guanti$cation of 6M? formation along with the degradation of M1" in crude en yme assays. "he reaction mi!ture contained "ris–H2I bu/er ,pH 4.0-, + mM M1", and 0.5 mg of crude en yme prepared from M1"-grown NyZ550 cells.




' i(u e , . %roposed M1" degradation pathway and involved genes in Aminobacter sp. strain NyZ550. ,?- >ene clusters involved in catabolism of M1". "he transcriptional levels of genes in the M1" degradation pathway are upregulated, as indicated by the log foldchange values upon each gene. C2, foldchange. 2lusters + and . encompass the methylamine catabolic genes. "mm, trimethylamine monoo!ygenase0 "dm, trimethylamine N-o!ide demethylase0 DmmABC, 6M? monoo!ygenase0 >mas, -glutamylmethylamide synthetase0 Mgs?#2, N-methylglutamate synthase0 and Mgd?#26, N-methylglutamate dehydrogenase. 2luster ' contains the genes encoding the nic*el incorporation proteins , Hyp?#- and agmatinase. ,#- %roposed M1" degradation pathway in strain NyZ550.

the dead-end product >9?, but the e!act intermediates for supporting the growth of NyZ550 are un*nown. "wo possible pathways for bacterial transformation of M1" to >9? were proposed ,Cigure : 5-. ) ne was initiali ed by demethylation, with the intermediates including +-methylbiguanide and biguanide. ?lthough +-methylbiguanide was previously detected in batch e!periments with activated sludge from &&"%s,[7] in this study, it was not observed in growth or biotransformation assays when compared to standard in 82-G") C M: analysis. ?nother possible pathway was the direct hydrolysis of M1" with the probable release of 6M?. "herefore, we performed an en ymatic assay to detect 6M? using the crude en yme prepared from M1"-grown NyZ550 cells. "he activity of the crude en yme on M1" was con$rmed by the decrease in the characteristic absorption of M1" at .'' nm during the en ymatic reaction ,Cigure : 3?,#-, as well as the formation of product >9? in the reaction mi!ture ,Cigure : 32,6-. Most importantly, a signi$cant amount of 6M? was detected in the reaction mi!tures by ion chromatography ,Cigure : 4- and >2–M: ,Cigure : 7-. 2onsumption of +.0. ± 0.0' mM M1" led to the formation of +.+. ± 0.+4 mM 6M? in the reaction mi!ture within 3. h ,Cigure (#-. 6M? was a favorable growth substrate for strain NyZ550 but >9? was not ,Cigure '-. Curthermore, we observed that an e0uivalent amount of 6M? and M1" resulted in an e0ual biomass accumulation of strain NyZ550 ,Cigure : +0-, indicating that 6M? was the only intermediate supporting bacterial growth.

,.8. /enes $n4o!4ed in M'T Degradation in Strain NyZ550. "o further elucidate the degradation mechanism of M1" by strain NyZ550, complete genome and comparative transcriptome analyses were carried out to identify the 61>s in response to M1". ? total of 544' e!pressed genes were detected, and of them, +03 genes were upregulated, and +0( genes were downregulated ,Cigure : ++?-. J1>> analysis showed that the upregulated genes were signi$cantly enriched ,p K 0.0+- in pathways of photosynthesis, ;agellar assembly, ribosome, glyo!ylate and dicarbo!ylate metabolism, methane metabolism, one-carbon pool by folate, and glycine, serine, and threonine metabolism ,Cigure : ++#-. In addition, >) annotation analysis also showed that the upregulated genes were enriched in the tricarbo!ylic acid cycle and one-carbon metabolism, implying that these processes were li*ely to participate in M1" degradation.

3.8.1. Genes Involved in the MET Degradation Pathway of Strain NyZ !. #ased on the genomic annotation, there were two gene clusters in the genome of strain NyZ550 encoding the methylamines degradation ,Cigure 5?-. ) ne of the clusters encoded the en ymes for catabolism of trimethylamine['7,(0] ,"mm, trimethylamine monoo!ygenase and "dm, trimethylamine N-o!ide demethylase- and 6M?[(+] ,6mm?#2, 6M? monoo!ygenase-, while another cluster encoded the en ymes for catabolism of methylamine[(.] ,>mas, -glutamylmethylamide synthetase0 Mgs?#2, N-methylglutamate synthase0 and Mgd?#26, N-methylglutamate dehydrogenase-. "he transcriptome data showed that the transcription of dmm, gmas, mgs, and mgd genes was highly induced during the growth of strain NyZ550 on M1" ,"able : '-, indicating that they encoded the en ymes responsible for the catabolism of 6M?, a catabolic intermediate of M1". Moreover, assimilation of 6M? in strain NyZ550 was most li*ely coupled to the serine cycle ,Cigure : +. - due to the signi$cant induction of its pathway genes and formate-tetrahydrofolate catabolic genes ,"able : '-.

"ill date, the gene encoding the en yme for hydrolysis of M1" has not been identi$ed. &e analy ed the proteins encoded by the +03 upregulated genes in the Inter%ro protein families and domains database[(] to screen potential M1" hydrolases. It turned out that a putative agmatinase was attractive due to its potential role in hydrolysis of the 2–N bond. ?gmatinase cataly es the cleavage of the 2–N bond of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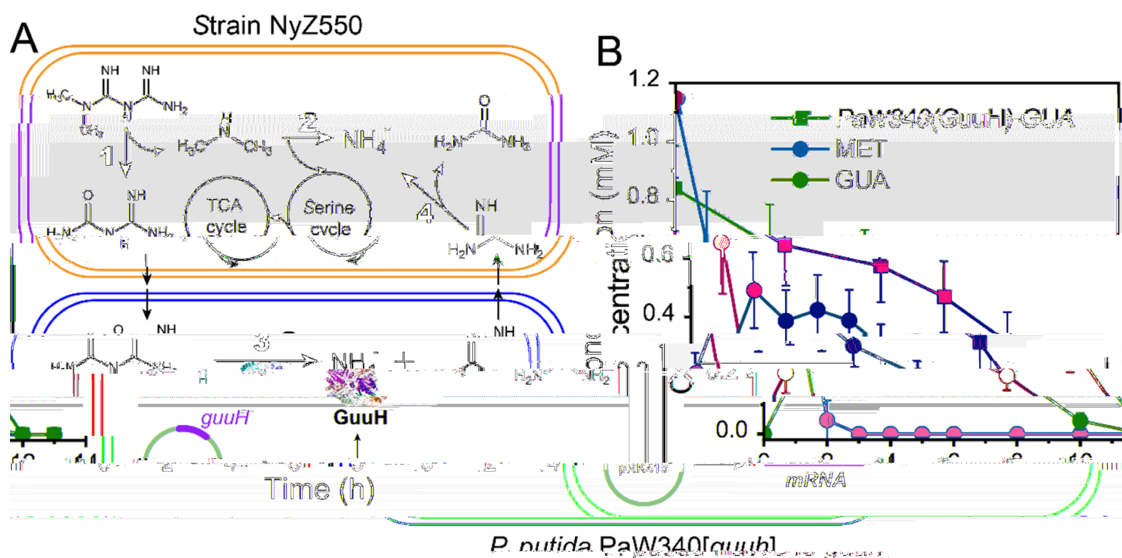


' i(u e -. 2omplete degradation of M1" and >9? by a synthetic co-culture. ,?- : chematic diagram for the roles of the bacterial strains in the mi!ture. " he *ey processes in M1" and >9? degradation are shown@ ,+- hydrolysis of M1"0 ,. - o!idative degradation of 6M?0 ,' - hydrolysis of >9?0 and ,(- hydrolysis of guanidine. ,#- 6egradation of M1" and >9? by the mi!ture.

agmatine with the formation of putrescine and urea. In this reaction, an o!ygen atom from H.) is incorporated into urea, which is similar to the reaction mechanism for hydrolysis of M1". ?gmatinases are reported to be nic*el or manganese-dependent hydrolase. " wo genes encoding the nic*el incorporation proteins ,Hyp?#- which is re0uired for the loading of divalent metal ion on agmatinase are ad0acent to the upregulated agmatinase gene in strain NyZ550 ,Cigure 5?-. Curther investigations of M1" hydrolase in biochemistry, catalytic mechanism, evolution, and ecological distribution would be helpful for a better understanding of the environmental fate of M1". #ased on the metabolite analyses and results from transcriptomic se0uencing, the degradation mechanism of M1" and its intermediates by strain NyZ550 was inferred, as shown in Cigure 5#.

>9? hydrolase was absent from the NyZ550 genome, which is consistent with the accumulation of >9? during degradation of M1". ?lthough >9? was not degraded by strain NyZ550, guanidine, a *nown catabolic intermediate of >9?,[' ·] was readily utili ed as a nitrogen source by strain NyZ550 ,Cigure '#-. ? putative arginase of strain NyZ550 was homologous to the guanidinase >dmH ,5+< identity- from cyanobacterium Synechocystis sp. %22 540'[((,(5] li*ely cataly ed the conversion of guanidine into ammonium and urea.

3.8.". #ther I$%ortant &iological Processes ' elated to MET Degradation. M1" is a hydrophilic compound and its degradation by strain NyZ550 is dependent on intracellular en ymes, and therefore, M1" must be transported into the cell before it can be degraded. " his process mainly involves energy metabolism, signal sensing, binding, and transmembrane transportation. ?s shown in " able :(, some bacterial chemota!is genes were upregulated during growth on M1", which is bene$cial for accessing and transporting substrates. >enes encoding ?#2 transporters and substrate-binding proteins were also upregulated, suggesting that they are li*ely involved in the transmembrane transportation of M1". ?dditionally, an upregulated gene encoding the putative guanidinium e!porter is li*ely contributed to minimi ing the stress e!erted by e!ogenous M1" or its catabolic products.

,.:. Degradation of M' T and /%A by a Constructed -acteria! Mi2ture. 2onsidering the ris* of fre0uently detected >9? accumulation in the environment ," able +-[· · (] and the accumulation of the dead-end product >9? in the degradation of M1" by strain NyZ550 in this study, >9? degradation is also necessary for bioremediation of M1"-contaminated sites. " he results here regarding M1" catabolism and the genes involved in strain NyZ550 clearly indicated that the conversion of >9? was the catabolic bottlenec*. " hus, a bacterial mi!ture was constructed for complete degradation of M1" and >9?, consisting of strain NyZ550 and P. putida %a&'(0 harboring a >9? hydrolase gene ,guuH- ,Cigure 5?-. >uuH converted >9? into guanidine,[' ·] which was subse0uently utili ed as a nitrogen source by strain NyZ550 ,Cigure '#-. P. putida %a&'(0 cells e!pressing >9? hydrolase ,designated P. putida %a&'(0-0>uuHP- degraded >9? at a rate of +.5 nmol mg protein$^{-+}$ h$^{-+}$ ,Cigure 5#-. " he bacterial mi!ture composed of strain NyZ550 and P. putida %a&'(00>uuHP e!hibited the capacity to eliminate both M1" and >9?. M1" ,+.. mM- disappeared within ' h, accompanied by the signi$cant accumulation of >9? with a ma!imum concentration of 0.5 mM at . h. 2omplete degradation of >9? was observed after +. h ,Cigure 5#-.

" he constructed bacterial mi!ture capable of complete degradation of M1" and >9? in water provides a potential strategy for bioremediation of M1"-contaminated environments. " he degradation of >9? appears to be the rate-limiting step for M1" degradation in the environment, due to the fact that it is often detected as a dead-end product of M1". " he results presented here suggest that the introduction of a >9? degrader can be a potential solution for accelerating complete removal of M1" contamination through the combined e/ect of indigenous M1" degraders in &&"%s, such as strain NyZ550.

,.#0. $mp!ications for M' T0Contaminated ' n4iron0 ments. 1nvironmental e!posure to M1" is increasingly



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recogni ed as an emerging issue threatening human health and ecological safety.[+3,+4,. 5,(5] 2learly, microorganisms are the vital drivers of M1" degradation in the environment. Here, based on an investigation of M1" contamination in wastewater from &&"%s and the M1" degradation capacity of microorganisms from these wastewater samples, we isolated and characteri ed a M1"-degrading bacterium Aminobacter sp. strain NyZ550. 2urrently, the primary biodegradation pathways for M1" are predicted based on identifying the M1" metabolites in situ or derived from degradation by microbial consortia.[+7,.0] ) ne of the possible degradation pathways involves the se0uential removal of two urea, giving the $rst intermediate dimethylguanidine and subse0uently 6M? ,Cigure :5-. ?nother conceivable pathway results in the production of biguanide through double deal*ylation and further formation of >9? as a dead-end product[7] ,Cigure :5-. #ased on the results in this study and comparison with previously predicted pathways, a novel pathway for partial catabolism of M1" in strain NyZ550 was proposed ,Cigure 5#-. In this pathway, M1" was directly hydroly ed to produce >9? and 6M?. ?lthough >9? was not cataboli ed further, 6M? was assimilated through a complete set of en ymes for methylamine degradation in strain NyZ550 ,Cigure 5-, which is echoed by the fact that strain NyZ550 was able to grow on 6M?, as shown in Cigure ʼ?. "he results support the observations of fre0uent accumulation of >9? in the environment[ʼ,(] and suggest a possible role of this pathway in environmental transformation of M1". In the future, functional characteri ation of the catabolic en ymes involved in M1" degradation at the genetic, biochemical, and ecological levels would broaden our understanding of the environmental fate of the widely used antidiabetic drug M1".

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### * Supporting $nformation

"he :upporting Information is available free of charge at https@FFpubs.acs.orgFdoiF+0.+0. +Facs.est.. c03557.

6etails of growth, biotransformation product identi$cation, and transcriptome ,%6C-

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### Notes

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Recommended by ACS

(IIHFWV RI WKH 'HVLFFDWLRQ 'XUDWLRQ R 5HVSRQVHV RI %LRILOP 0HWDEROLF $FWLY

/LQJ]KDQ 0LDR *HWXDQ +RX
+"/6"3:
&/7*30/.&/5"- 4$*&/$& 5&$)/0-0(:

0LFUREELDO 7UDQVIRUPDWLRQ RI 'LVVROYH GXULQJ WKH 2[LF 3URFHVV LQ )XOO36FD :DVWHZDWHU 7UHDWPHQW 3ODQWV

&DLIHQJ /LX +RQJTLDQJ 5HQ
+"/6"3:
&/7*30/.&/5"- 4$*&/$& 5&$)/0-0(:

1DWLRQDO :DVWHZDWHU 5HFRQQDLVVDQFH &RQVXPSWLRQ LQ $XVWUDOLD

)DKDG $KPHG .HYLQ 9 7KRPDV
+"/6"3:
&/7*30/.&/5"- 4$*&/$& 5&$)/0-0(:

$QWLELRWLF 3ROOXWLRQ RI 3ODQNWRQLF )RFXVHG RQ &RPPXQLW\ $QDO\VLV DQG WK /LQNLQJ ,QGLYLGXDO3 DQG &RPPXQLW\3/H

0 ' . /DNPDOL *XQDWKLODND <LQJ 3DQ
+"/6"3:
&/7*30/.&/5"- 4$*&/$& 5&$)/0-0(:

*HW 0RUH 6XJJHVWLRQV !



https://"oi.org/10.1021/acs.est.2c07##9
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