Isolation and Identification of Poly(3-Hydroxyvalerate)-Degrading Strains of *Pseudomonas lemoignei*

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By using selective enrichment of polyhydroxyalkanoate-degrading bacteria and poly(3-hydroxyvalerate)-containing granules from *Chromobacterium violaceum* as the carbon source, 10 new *Pseudomonas lemoignei* strains were isolated; these strains were able to degrade poly(3-hydroxyvalerate), as well as poly(3-hydroxybutyrate), in vitro. The new isolates were characterized and identified by comparing them with *P. lemoignei* LMG 2207^T (T = type strain). Like *P. lemoignei* LMG 2207^T cells, the cells of the 10 new isolates contained mainly hexadecenoic, hexadecanoic, octadecenoic, and dodecanoic acids, as well as hydroxylated fatty acids, and exhibited respiration in the presence of methylpyruvate, 3-hydroxybutyrate, and 4-hydroxybutyrate, but not in the presence of the 92 other carbon sources included in Biolog GN microplates. The protein patterns of the new isolates were almost identical to each other and very similar to the protein pattern of *P. lemoignei* LMG 2207^T. Some of the new isolates, but not *P. lemoignei* LMG 2207^T, contained megaplasmids that were about 200 kbp long. The 16S ribosomal DNA genes of strain A62, a representative of the 10 new isolates, and of *P. lemoignei* LMG 2207^T exhibited more than 0.99 sequence similarity. The DNA-DNA reassociation value for two representative strains was 100%, and the levels of DNA-DNA reassociation between these strains and the type strain were 60 and 61%. The taxonomy of *P. lemoignei* is briefly discussed.

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters that are synthesized and accumulated intracellularly under limiting growth conditions by a large variety of bacteria. Besides poly(3-hydroxybutyrate) [P(3HB)], polymers incorporating monomers other than 3-hydroxybutyrate have been detected. The monomer composition of the PHAs depends on the bacterial strain, as well as on the carbon sources supplied. The production of PHAs has been reviewed previously (2, 11, 39, 41). Because of their biodegradability, thermoplastic properties, and synthesis from renewable resources, PHAs are of biotechnological interest, and P(3HB) and copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (3) have been commercialized as Biopol, an alternative to petrochemically derived, nondegradable plastics. The biodegradability of Biopol has been demonstrated in different natural environments, and a large variety of PHA-degrading microorganisms, including gram-negative and gram-positive bacteria, streptomycetes, and molds, have been isolated (5, 14, 24, 25, 27–29, 33, 34). One of the first P(3HB)-degrading microorganisms identified was isolated more than 30 years ago and was named *Pseudomonas lemoignei* (7). Three *P. lemoignei* strains were the predominant microorganisms after enrichment in liquid medium containing P(3HB) granules. Only the type strain of this taxon has been preserved, and no PHA-degrading *P. lemoignei* strains have been isolated since the first isolation. The type strain of the species is particularly interesting, since it produces at least five different extracellular depolymerases (13), one of which is specifically synthesized during growth on poly(3-hydroxyvalerate) [P(3HV)] or valerate (30). Other PHA-degrading bacteria apparently synthesize only one depolymerase (13). The type

strain of *P. lemoignei* is specialized and utilizes homo- and copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate, their hydrolysis products, and a few organic acids (pyruvate, acetate, butyrate, valerate, succinate), but it is not able to grow on sugars, amino acids, or complex media (7).

In this paper a procedure for selective enrichment and isolation of PHA-degrading *P. lemoignei* strains, in which P(3HV) granules are used as the carbon source, is described. Ten new *P. lemoignei* isolates were identified and characterized by using fatty acid analysis, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of whole-cell proteins, the Biolog breathprinting system, 16S ribosomal DNA (rDNA) gene sequence analysis, DNA base composition analysis, DNA-DNA hybridization analysis, plasmid analysis, and conventional phenotypic characterization techniques.

MATERIALS AND METHODS

Isolation of P(3HV)-degrading microorganisms. To enrich for P(3HV)-degrading microorganisms, soil or compost that was collected in or near Göttingen, Germany, was placed in boxes and supplemented with PHAs. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) was added as buried Biopol bottles (Wella AG, Darmstadt, Germany), while P(3HV) was repeatedly added as broken cells of *Chromobacterium violaceum* DSM 30191 which had been cultivated under conditions that promoted polymer accumulation (40). The boxes were incubated at room temperature (18 to 25°C) or 30°C for several weeks. Diluted soil or compost suspensions in 0.9% (wt/vol) NaCl were used as inocula for 10-ml liquid cultures of mineral medium (35) supplemented with 0.15% (wt/vol) P(3HV) as the sole source of carbon and energy (14). After several days of aerobic incubation, clearing of the opaque P(3HV) suspensions was observed in most of the cultures. After a second incubation step performed with fresh medium under the same conditions, dilutions were streaked onto solid mineral medium supplemented with 0.3% (wt/vol) P(3HV) as the sole carbon source. P(3HV)-degrading bacteria were recognized by the appearance of clear halos surrounding the colonies (14) and were purified by alternate plating on mineral medium containing either P(3HV) or sodium valerate (0.15%, wt/vol). The 10 strains obtained were deposited in the Laboratorium voor Microbiologie Gent Culture Collection (LMG), Ghent, Belgium, as *P. lemoignei* strains. *P. lemoignei* LMG 2207^T (T = type strain) was obtained from LMG.

PHA accumulation. PHA accumulation was determined by performing a gas chromatographic analysis of the corresponding 3-hydroxymethyl esters of lyophilized cells (4).

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Fatty acid analysis. Strains were grown on Trypticase soy broth (BBL) supplemented with 1.5% (wt/vol) Bacto Agar (Difco) and 0.1% (wt/vol) sodium-DL-3-hydroxybutyrate (Serva) for 3 days at 28°C. Fatty acid methyl esters were prepared and separated by gas-liquid chromatography as described previously (1), with some modifications (26). The Microbial Identification System (Microbial ID, Inc., Newark, Del.) software package was used to identify the fatty acids. The same software package was used to cluster the fatty acid methyl ester profiles by the unweighted pair group method of averages (37) of Euclidean distances, which were calculated for each pair of profiles.

Breathprinting with the Biolog system. Strains were grown on Delafield medium (7) supplemented with 0.3% (wt/vol) 3-hydroxybutyrate for 2 days at 28°C. Suspensions were prepared, and Biolog GN microplates (Biolog, Inc., Hayward, Calif.) were inoculated as recommended by the manufacturer. After 3 days of incubation at 28°C, the plates were read visually, and wells showing any color reaction, compared with the control well were interpreted as positive.

Plasmid analysis. A plasmid analysis was performed by the method of Kado and Liu (16). Plasmid preparations were loaded onto an 0.8% (wt/vol) agarose gel and stained with ethidium bromide after electrophoresis.

SDS-PAGE of whole-cell proteins. The bacterial cells in 2-ml stationary cultures grown with sodium valerate were harvested by centrifugation, resuspended in 0.2 ml of denaturation buffer, and boiled for 5 min. For SDS-PAGE, the method of Laemmli (19) was used. A 15- μ l portion of each extract was loaded onto an SDS-12% (wt/vol) polyacrylamide gel. After electrophoresis, the proteins were stained with Coomassie blue and compared visually.

16S rDNA gene sequencing. The strains were incubated for 7 days in 5-ml portions of Trypticase soy broth (1.7% [wt/vol] Bacto Tryptone [Difco], 0.3% [wt/vol] Bacto Soytone [Difco], 0.25% [wt/vol] Bacto Dextrose [Difco], 0.5% [wt/vol] NaCl, 0.25% [wt/vol] K_2HPO_4) supplemented with 0.1% (wt/vol) DL-3-hydroxybutyrate under aerobic conditions. The genomic DNA of each strain was extracted by the method of Wilson (43). The 16S rDNA was amplified by PCR as described by Karlson et al. (17). The PCR products were sequenced directly by using a Taq Dye Deoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.), and electrophoresis was performed with a model 373A automatic sequencer (Applied Biosystems). The 16S rDNA sequences were aligned by using the sequence editing and analyzing program of Olsen (31) and reference sequences from members of the *Proteobacteria* obtained from the Ribosomal RNA Database Project (20). Conserved regions were used for the initial alignment. Alignment of variable regions was aided by the results of an analysis of the secondary structure. Overall similarity values, incorporating the Jukes and Cantor (15) correction factor, were calculated for sequence pair comparisons.

DNA base composition and DNA-DNA hybridizations. To prepare DNA, cells were grown at 28°C for 4 days in Roux flasks on minimal medium (7) supplemented with 0.3% (wt/vol) DL-3-hydroxybutyrate. DNA was prepared by the method of Marmur (22). The mean guanine-plus-cytosine (G+C) contents of DNAs were determined by the thermal denaturation method (8, 23). Levels of DNA-DNA binding, expressed as percentages, were determined spectrophotometrically by the initial renaturation rate method of De Ley et al. (9). Each value was the average of the values from two hybridization experiments. The total DNA concentration used was 59 μ g/ml, and the optimal renaturation temperature, calculated from the G+C content with the equation of De Ley (8), was 77.3°C in $2\times$ standard saline citrate buffer (SSC) ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7).

Electron microscopy. Cells grown on minimal medium (35) supplemented with 0.3% (wt/vol) DL-3-hydroxybutyrate were negatively stained with 3% phosphotungstate and examined with a Phillips model EM 301 electron microscope operated at 80 kV.

Nucleotide sequence accession numbers. The 16S rDNA gene sequences of *P. lemoignei* LMG 2207^T and strain A62 have been deposited in the EMBL data library under accession numbers X92555 and X92554, respectively.

RESULTS

Isolation of P(3HV)-degrading bacteria. After the enrichment procedure, 10 bacterial strains that are able to use P(3HV) as a sole source of carbon and energy were isolated on P(3HV)-containing media. Strains A51 (= LMG 16484), A52 (= LMG 16485), A53 (= LMG 16486), A58 (= LMG 16487), A60a (= LMG 16488), and A60b (= LMG 16489) were isolated from a box containing garden soil supplemented with broken Biopol bottles and incubated at 30°C. Strains A62 (= LMG 16480) and A64 (= LMG 16841) were isolated from a box containing soil from a meadow, and strains K21 (= LMG 16842) and K24 (= LMG 16843) were isolated from a box containing compost. Both of the latter boxes were supplemented with P(3HV)-containing *C. violaceum* cells and were incubated at room temperature. Like *P. lemoignei* LMG 2207^T, these gram-negative, oxidase- and catalase-positive isolates

FIG. 1. Electron micrograph of a negatively stained cell of strain A62. Bar = 1 μ m. (Prepared by M. Hoppert.)

grew slowly on solid mineral medium supplemented with valerate or 3-hydroxybutyrate, forming small, circular, cream-colored colonies which strongly adhered to the agar, and growth on butyrate (0.3%, wt/vol) was very poor. None of the new isolates or *P. lemoignei* LMG 2207^T grew on rich media like nutrient broth (Oxoid) or Luria-Bertani medium (1% [wt/vol] tryptone [Oxoid], 0.5% [wt/vol] yeast extract [Oxoid], 1% [wt/vol] NaCl; pH 7.5) or with sugars, such as glucose (0.5%, wt/vol) and fructose (0.5%, wt/vol), as sole carbon sources. However, all of the strains were capable of extracellular degradation of P(3HB) and P(3HV) and intracellular synthesis of P(3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate). In the presence of tyrosine (0.05%, wt/vol), a brown pigment was produced by the 10 new strains and *P. lemoignei* LMG 2207^T on media supplemented with P(3HB) but not on media supplemented with DL-3-hydroxybutyrate. Cells of all of these strains were motile, displaying alternating backward movement and forward movement. Electron micrographs of cells of strains A62 (Fig. 1), K24, and A51, as well as *P. lemoignei* LMG 2207^T (data not shown), revealed the presence of a single monopolar inserted flagellum and electron transparent inclusions that might be P(3HB) granules. When enrichment for P(3HV)-degrading microorganisms was omitted and dilutions of soil samples were directly plated onto P(3HV) agar plates, three P(3HV)-degrading actinomycetes and two gram-negative strains, which clearly differed from *P. lemoignei* LMG 2207^T, were isolated.

Characterization of the isolates by fatty acid analysis. The 10 new isolates were compared with *P. lemoignei* LMG 2207^T by performing a fatty acid analysis. The fatty acid methyl ester compositions of the strains are shown in Table 1. The dendrogram obtained by clustering the profiles is shown in Fig. 2. The fatty acid methyl ester profiles of the 10 new isolates were very similar to each other and resembled the profile of *P. lemoignei* LMG 2207^T. All of these organisms contain mainly hexadecenoic acid (16:1 ω 7 *cis*), hexadecanoic acid (16:0), octadecenoic acid (summed feature 7), and dodecanoic acid (12:0), as well as hydroxylated fatty acids (Table 1). The 10 new isolates clustered together at a Euclidean distance of 8 and grouped with the type strain at a Euclidean distance of 16. *P. lemoignei* LMG 2207^T differed slightly because of its higher content of octadecenoic acid (summed feature 7).

Breathprinting with the Biolog system. The metabolic breathprints of the 10 new isolates and *P. lemoignei* LMG

TABLE 1. Fatty acid methyl ester compositions of P(3HV)-degrading strains A51, A52, A53, A58, A60a, A60b, A62, A64, K21, and K24 and *P. lemoignei* LMG 2207^T

Fatty acid methyl ester	New isolates ^a		% of total fatty acids in <i>P. lemoignei</i> LMG 2207 ^T
	No. of strains ^b	% of total fatty acids	
		Mean SD	
10:0	10	1.0 0.1	ND ^c
12:0	10	7.1 0.3	5.3
14:0	10	2.6 0.1	1.3
16:0	10	17.2 0.9	12.8
18:0	3	0.3 0.5	ND
14:1 ω 5 ^d	5	0.4 0.5	ND
16:1 ω 7 <i>cis</i>	10	37.5 2.9	39.3
18:1 ω 9 <i>cis</i>	2	0.2 0.4	ND
17:0 cyclo ^d	9	1.9 1.6	1.9
10:0 3OH	10	6.1 0.7	3.6
12:0 3OH	10	8.2 0.3	5.7
14:0 2OH	10	6.3 0.6	4.7
Summed feature 7 ^e	10	11.2 0.8	25.5

^a Strains A51, A52, A53, A58, A60a, A60b, A62, A64, K21, and K24.

^b Number of strains that contain each fatty acid methyl ester.

^c ND, not detected.

^d The position of the double bond or cyclopropane ring is not known.

^e Fatty acids 18:1 ω 7 *cis*, 18:1 ω 9 *trans*, and 18:1 ω 12 *trans* cannot be separated gas chromatographically by the Microbial Identification System and together are designated summed feature 7.

FIG. 3. SDS-PAGE of whole-cell proteins of *P. lemoignei* strains. Lane 1, strain A51; lane 2, strain A52; lane 3, strain A53; lane 4, strain A58; lane 5, strain A60a; lane 6, strain K21; lane 7, strain K24; lane 8, strain A62; lane 9, strain A64; lane 10, *P. lemoignei* LMG 2207^T.

Plasmid analysis. The new isolates and *P. lemoignei* LMG 2207^T were analyzed to determine the presence of circular plasmids. No plasmids were detected in strains A64, K21, and K24 and *P. lemoignei* LMG 2207^T. Strains A51, A52, A53, A58, A60a, A60b, and A62 harbored a plasmid that was about 200 kbp long, and strains A51 and A58 contained an additional plasmid that was about 30 kbp long.

16S rDNA sequencing. The 16S rDNA genes from strain A62, a representative of the new *P. lemoignei* isolates, and from *P. lemoignei* LMG 2207^T were sequenced. The 16S rDNAs of strain A62 and *P. lemoignei* LMG 2207^T have a level of mutual sequence similarity and an overall level of similarity of 0.99. The sequences differ mainly between positions 73 and 97 (*Escherichia coli* numbering [6]). The overall levels of similarity of the 16S rDNA sequences of these strains and 19 other representatives of the *Proteobacteria* are shown in Table 2. The 16S rDNA sequence analysis of *P. lemoignei* LMG 2207^T and strain A62 revealed that these organisms belong to the beta subclass of the *Proteobacteria*. *P. lemoignei* LMG 2207^T and strain A62 exhibited 0.94 overall similarity with *Zoogloea ramigera* ATCC 25935, 0.92 similarity with *Burkholderia solanacearum*, and 0.91 similarity with *Alcaligenes eutrophus* and *Pseudomonas andropogonis* and levels of similarity lower than 0.91 with the other representatives of the *Proteobacteria*, including *Z. ramigera* ATCC 19544^T (Table 2).

DNA base composition and DNA-DNA hybridization. The DNAs of *P. lemoignei* LMG 2207^T and strains A62 and A52 had % G+C contents of 59.7, 58.9, and 59.5 mol%, respectively. The DNA-DNA binding ratios were 100% for strains A62 and A52, 61% for strain A62 and *P. lemoignei* LMG 2207^T, and 60% for strain A52 and *P. lemoignei* LMG 2207^T.

P. lemoignei was isolated and described more than 30 years ago and is recognized as one of the first known P(3HB)-degrading microorganisms (7). Of the three strains isolated originally, only one was preserved. Since then, numerous PHA-degrading microorganisms have been isolated, but none of them had the characteristics of *P. lemoignei*. It was presumed that this species is not representative of the PHA-degrading

2207^T were investigated by using Biolog GN microplates. All of the strains exhibited weak positive reactions in the wells containing methylpyruvate, 3-hydroxybutyrate, and 4-hydroxybutyrate. None of the strains produced color reactions in the presence of 28 carbohydrates, 22 other carboxylic acids, 20 amino acids, the polymers glycogen, α -cyclodextrin, dextrin, Tween 40, and Tween 80, the alcohols 2,3-butanediol and glycerol, the amides succinamic acid, glucuronamide, and alaninamide, the amines phenylethylamine, 2-aminoethanol, and putrescine, the aromatic chemicals inosine, urocanic acid, thymidine, and uridine, the phosphorylated chemicals DL- α -glycerol phosphate, glucose 1-phosphate, and glucose 6-phosphate, bromosuccinic acid, and monomethylsuccinate.

SDS-PAGE of whole-cell proteins. The protein patterns of the 10 new isolates were almost identical to each other and very similar to the protein pattern of *P. lemoignei* LMG 2207^T (Fig. 3).

FIG. 2. Dendrogram grouping strains A51, A52, A53, A58, A60a, A60b, A62, A64, K21, and K24 and *P. lemoignei* LMG 2207^T on the basis of their fatty acid methyl ester compositions.

TABLE 2. Overall levels of similarity between the 16S rDNA sequences of *P. lemoignei* LMG 2207^T and strain A62 and the 16S rDNA sequences of 19 representatives of the beta subclass of the *Proteobacteria* and *E. coli*

Species	Strain	EMBL accession no.	Level of similarity to:	
			Strain LMG 2207 ^T	Strain A62
<i>Pseudomonas lemoignei</i>	LMG 2207 ^T	X92554	1.00	0.99
<i>Pseudomonas lemoignei</i>	A62	X92555	0.99	1.00
<i>Zoogloea ramigera</i>	ATCC 25935	X74914	0.94	0.94
<i>Zoogloea ramigera</i>	ATCC 19544 ^T	X74913	0.90	0.89
<i>Burkholderia solanacearum</i>	ATCC 11696 ^T	X67036	0.92	0.92
<i>Burkholderia cepacia</i>	ATCC 25416 ^T	M22518	0.90	0.90
<i>Alcaligenes eutrophus</i>	Unknown	M32021	0.91	0.91
<i>Alcaligenes faecalis</i>	Unknown	M22467	0.90	0.90
<i>Pseudomonas andropogonis</i>	ATCC 23061	X67037	0.91	0.91
<i>Rhodocyclus tenuis</i>	DSM 109 ^T	D16208	0.90	0.90
<i>Rubrivivax gelatinosus</i>	ATCC 17011 ^T	M34132	0.89	0.90
<i>Comamonas testosteroni</i>	ATCC 11996 ^T	M11224	0.89	0.89
<i>Nitrosomonas europaea</i>	Unknown	— ^a	0.88	0.89
<i>Neisseria gonorrhoeae</i>	Unknown	M34130	0.88	0.88
<i>Azoarcus indiges</i>	VB32	L15531	0.88	0.88
<i>Methylophilus methylotrophus</i>	ATCC 53528 ^T	L15475	0.88	0.88
<i>Escherichia coli</i>	Unknown	— ^b	0.82	0.81

^a See reference 32.

^b See reference 6.

microflora that occurs in the environment (27). By using an enrichment procedure in which P(3HV) was the sole source of carbon and energy, we succeeded in isolating from soils 10 strains that are able to degrade P(3HV) and P(3HB) in vitro and have cultural characteristics very similar to those of *P. lemoignei* LMG 2207^T. Such strains could not be isolated when the enrichment procedure was omitted, and no other P(3HV)-degrading strains were isolated after enrichment. This indicates that the enrichment procedure in which P(3HV) granules are used as the sole source of carbon and energy seems to be especially appropriate for the isolation of *P. lemoignei* strains from soils.

To verify that the new isolates were *P. lemoignei* strains, they were compared with *P. lemoignei* LMG 2207^T by performing fatty acid, protein, and plasmid analyses and by using breath-printing. The results clearly showed that the P(3HV)-degrading strains isolated in this study are phenotypically almost identical to each other and very similar to *P. lemoignei* LMG 2207^T. The G+C contents of the DNAs of strains A52 and A62 are very similar to the value obtained for *P. lemoignei* LMG 2207^T (59.7 mol%) and the value published previously for *P. lemoignei* LMG 2207^T by Delafield et al. (7) (58.2 mol%). A close phylogenetic relationship between the 10 new isolates and *P. lemoignei* was demonstrated by aligning the 16S rDNA sequences of representative strain A62 and *P. lemoignei* LMG 2207^T; a level of 16S rDNA sequence similarity of more than 0.99 was obtained. The levels of hybridization between strain A52 and A62 DNAs and *P. lemoignei* LMG 2207^T DNA were 60 and 61%, respectively. Therefore, we identified strains A51, A52, A53, A58, A60a, A60b, A62, A64, K21, and K24 as *P. lemoignei* strains.

P. lemoignei is not an authentic member of the genus *Pseudomonas*, whose members belong to the gamma subclass of the *Proteobacteria* (38). It has been shown by rRNA-DNA hybridization that *P. lemoignei* is phylogenetically related to rRNA group III of De Vos and De Ley (10). This group constitutes

one of the branches within the beta subclass of the *Proteobacteria* (38) and includes microorganisms now classified as *Burkholderia* sp. and *A. eutrophus*. This finding is supported by our phylogenetic data. Strain A62 and *P. lemoignei* LMG 2207^T exhibited more than 0.91 overall similarity in their 16S rDNA sequences to *B. solanacearum* and *A. eutrophus* and 0.94 overall similarity to *Z. ramigera* ATCC 25935. Strain ATCC 25935 (= IAM 12670) is not an authentic *Zoogloea* strain and is related to *Burkholderia cepacia* and *A. eutrophus* (36). However, the taxonomic homogeneity of the genus *Burkholderia* has been questioned, and the removal of *B. solanacearum* and *Burkholderia pickettii* from the genus has been proposed (12, 21). In contrast to *Burkholderia* species (including *B. solanacearum* and *B. pickettii*) (12) and *A. eutrophus* (18), *P. lemoignei* is able to grow only on a very limited number of substrates. The hydroxylated fatty acid fraction of *P. lemoignei* cells contains 3-hydroxydecanoic acid (10:0 3OH), 3-hydroxydodecanoic acid (12:0 3OH), and 2-hydroxytetradecanoic acid (14:0 2OH), while *Burkholderia* cells (including *B. solanacearum* and *B. pickettii* cells) typically contain 3-hydroxytetradecanoic acid (14:0 3OH) (12, 42, 44; this study). *P. lemoignei* may constitute a separate phylogenetic branch within the beta subclass of the *Proteobacteria*, but its phylogenetic relationships with other members of this branch have to be scrutinized further before any nomenclatural proposal can be formulated.

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