Rhodamine 123 efflux transporter in *Haloferax volcanii* is induced when cultured under 'metabolic stress' by amino acids: the efflux system resembles that in a doxorubicin-resistant mutant

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In this paper, we report that an archaebacterium, *Haloferax volcanii*, cultured in medium containing a large excess of amino acids showed very low levels of rhodamine 123 (RH123), which is a potent substrate for P-glycoprotein and the bacterial multidrug efflux transporter. This low level involved the active efflux of RH123 from the cells. The level of intracellular RH123 was increased and the efflux inhibited by the Ca^{2+}-channel antagonist verapamil and also by various anti-cancer drugs. The efflux transporter was suggested to be ATP-driven. We have previously selected a mutant of *H. volcanii* with resistance to doxorubicin, by repeatedly culturing cells in 1.5 μM doxorubicin [Miyauchi, Komatsubara and Kamo (1992) Biochim. Biophys. Acta **1110**, 144–150]. The acquisition of resistance to doxorubicin involves the active expulsion of lipophilic drugs such as RH123 and doxorubicin. It is notable that the drug spectrum and ATP-dependency of the amino acid-induced efflux transporter resemble those of the efflux transporter induced by doxorubicin.

INTRODUCTION

We selected a mutant from the archaebacterium *Haloferax volcanii* that acquired resistance to doxorubicin (DOX) which involved the ATP-driven extrusion of DOX ([1]; M. Komatsubara, A. Abe, R. Okumura, S. Miyauchi and N. Kamo, unpublished work). This efflux system transported not only DOX but also structurally unrelated lipophilic compounds such as vinblastine, vincristine, ethidium bromide and monensin (M. Komatsubara, A. Abe, R. Okumura, S. Miyauchi and N. Kamo, unpublished work). The Ca^{2+}-channel antagonist verapamil was a potent modulator of this transporter. These properties resembled those of P-glycoprotein in mammalian cells.

The DOX-efflux transporter can also be detected unequivocally in wild-type cells [1]. A question arises as to the physiological function of the efflux transporter. The strategic transport direction and the recognition of structurally unrelated toxic compounds implies that the efflux transporter plays a role in actively expelling cytotoxic compounds or toxic metabolites generated in cells, thus protecting them [3,4].

We also found that when wild-type cells were cultured in normal medium supplemented with a high concentration of glucose or fructose, these cells acquired resistance to DOX which involved the activation of the efflux transporter (S. Tanabu, E. Imai, A. Abe, R. Okumura, K. Kaidoh, S. Miyauchi, T. Nara and N. Kamo, unpublished work). In contrast, an excess of the non-metabolizable glucose analogue, 2-deoxyglucose, or 3-*O*-methylglucose did not enhance the activity of the efflux transporter, implying the involvement of glucose or fructose metabolism. These results suggested that glucose or fructose metabolism produced some substances which (i) were cytotoxic, (ii) upset the homoeostasis of the cell or (iii) prevented growth. Under these circumstances, cells activate the efflux transporter to remove the substances and protect themselves [3,4].

Using the potent substrate for the efflux system, rhodamine 123 (RH123), we investigated whether other nutrients activated the system. In this study, we have shown that an excess of amino acids in normal culture media can also activate the efflux transporter. These properties resemble those of the DOX-efflux transporter in the mutant.

MATERIALS AND METHODS

Chemicals

Verapamil, daunomycin, vinblastine, vincristine, colchicine and *N*,*N*′-dicyclohexylcarbodiimide (DCCD) were purchased from Sigma (St. Louis, MO, U.S.A.). RH123 and bis(1,3-dibutylbarbituric acid)trimethine oxonol (OXO) were purchased from Molecular Probes (Eugene, OR, U.S.A.). DOX was supplied by Kyowa Hakko Kogyo (Tokyo, Japan). All other reagents were commercial products of analytical grade.

Cell culture and cell preparation

Haloferax volcanii was obtained from the National Collections of Industrial and Marine Bacteria (Aberdeen, U.K.). Cells were grown under the same conditions as described previously [1]. When necessary, excess amounts of amino acids were added to the normal culture medium. Cells in late-logarithmic growth phase were harvested as reported. Protein concentration was measured by the method of Lowry et al. [6] using BSA as the standard.

RH123 accumulation and efflux

Since fluorescent RH123 is recognized by the DOX efflux transporter [1], RH123 accumulation was determined by flow cytometry to estimate the activities of the transporter. Methods were essentially as described elsewhere [1]. Briefly, a cell suspension was diluted to 0.1 mg of protein/ml in medium con-

Abbreviations used: DCCD, *N,N*′-dicyclohexylcarbodiimide; DOX, doxorubicin; OXO, bis(1,3-dibutylbarbituric acid)trimethine oxonol; RH123, rhodamine 123.

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taining 4 M NaCl buffered with 50 mM Mops at pH 7.0, to which arginine and glutamate (final concentrations, 5 mM) were added as energy sources. The medium containing 4 M NaCl and 50 mM Mops (pH 7.0) is referred to herein as uptake buffer. After an incubation for 5 min at 25 °C, the fluorophore, RH123 (final concentration, 0.22 μM), was added. The RH123 accumulation reached equilibration within 10–15 min [1]. An aliquot of cell suspension (500 μl) was taken after equilibrium, and the intracellular RH123 concentration was measured using an EPICS CS flow cytometer (Coulter Electronics, Hialeah, FL, U.S.A.) at an excitation wavelength of 488 nm (bandwidth, $\pm$5 nm). Fluorescence emission was detected through a bandpass filter (520$\pm$5 nm).

RH123 efflux from the cells started when the loaded cell suspension was diluted. RH123 (8 μM) was loaded into cells in the presence or absence of 100 μM verapamil. After 15 min, an aliquot (1 ml) of the suspension was diluted in RH123-free uptake buffer (10 ml). RH123 remaining in the cells was determined with the flow cytometer at various times.

Membrane-potential estimation

The membrane potential was estimated using the fluorescent probe OXO, which is not a substrate for the DOX efflux transporter [1]. The intracellular fluorescence intensity was measured with the flow cytometer as described for RH123. OXO fluorescence intensity was prompted in cells by membrane depolarization with 1×10^{-4}% Triton, the change in OXO fluorescence reflecting that in the membrane potential (see the Results and Discussion section).

Intracellular ATP level

Cell suspensions (2 mg of protein/ml) in the uptake buffer were incubated without stirring in the presence of various concentrations of DCCD (10–150 μM), which is a H^+-ATPase inhibitor, at 4 °C, overnight (M. Komatsubara, A. Abe, R. Okumura, S. Miyauchi and N. Kamo, unpublished work). Cells were then centrifuged at 9000 ***g*** for 10 min and resuspended in DCCD-free uptake buffer, to which 5 mM arginine and 5 mM glutamate were added. The protein concentration was adjusted to 0.1 mg/ml. After an incubation at 25 °C for 15 min, a 0.5 ml aliquot of the suspension was centrifuged and the precipitate was mixed with 0.7 ml of 4.2% $HClO_4$. After neutralization with 0.64 M KOH and centrifugation at 9000 ***g*** for 10 min, the ATP concentration in the supernatant was determined using a J4-7741 Chem-Glo Photometer (American Instrument, Silver Spring, MD, U.S.A.), with firefly lantern extract (Sigma, MO, U.S.A.).

RESULTS AND DISCUSSION

Cells cultured in a large excess of amino acids accumulate low levels of RH123 due to a verapamil-inhibitable efflux system

Figure 1 shows the steady-state RH123 accumulation by the cells cultured in the presence of various concentrations of leucine and alanine. Amino acids in the culture medium reduced levels of RH123 in a concentration-dependent manner, with minimal values at approx. 30 mM. To determine whether the efflux was responsible for the low level of RH123 accumulation, RH123 efflux was measured. Figure 2 shows that RH123 was rapidly expelled from the cells cultured in the presence of 50 mM leucine, over a period of approx. 10 min. The rapid efflux was completely abolished by the Ca^{2+}-channel antagonist verapamil (100 μM), whereas the efflux from cells cultured in the normal medium was minimal. To confirm whether verapamil directly affected the efflux system, we monitored the intracellular ATP level and membrane potential. The change in membrane potential was estimated using a membrane-potential probe, OXO. The intracellular ATP level, as well as the membrane-potential change,

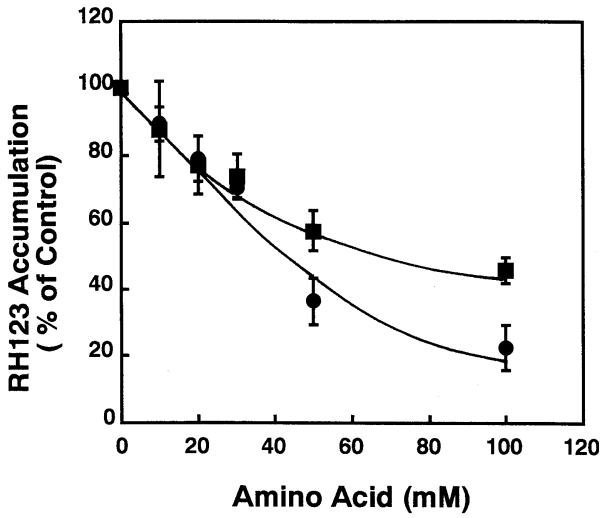

Figure 1 Effect of amino acids in the culture medium on RH123 accumulation

Cells were cultured in normal medium containing various concentrations of leucine (●) and alanine (■), then harvested at the late-log phase. After an incubation with 0.22 μM RH123 in uptake buffer containing 5 mM arginine plus glutamate for 15 min at 25 °C, the amount of RH123 accumulated in cells was determined by flow cytometry. The ordinate represents the percentage of fluorescence intensity with reference to the RH123 fluorescence of cells cultured in the normal medium. Each point represents the mean$\pm$S.E.M. of six determinations.

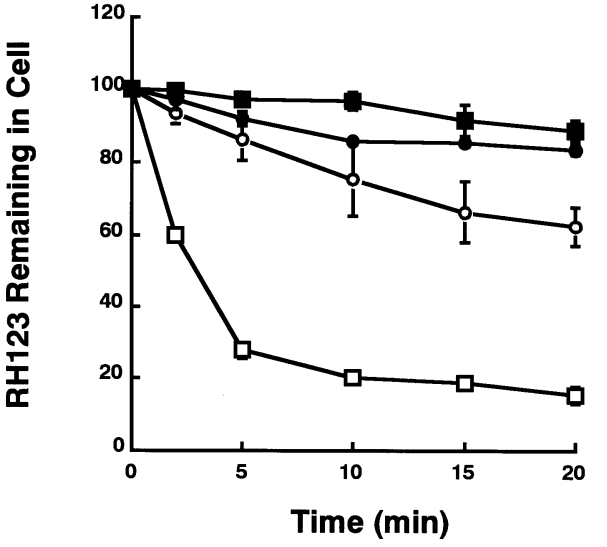

Figure 2 Efflux of RH123 from the cells cultured in medium containing 50 mM leucine

Cells were incubated with RH123 (8 μM) for 15 min. Thereafter, an aliquot of cell suspension (0.5 ml) was diluted with 10 ml of the RH123-free uptake buffer with (closed symbols) or without (open symbols) 100 μM verapamil. The amount of RH123 remaining in the cells was expressed as a percentage of the level in the cells before dilution. Circles and squares represent the amounts of RH123 in cells cultured in the normal medium and that containing 50 mM leucine, respectively. Each point represents the mean$\pm$S.E.M. of three determinations.

Table 1 Effect of pharmacological agents on membrane potential

The experimental medium and the conditions were the same as those described in the legend to Figure 1 except for the addition of 0.05 μM OXO, a fluorescent membrane-potential probe. The intracellular fluorescence intensity of OXO was determined by flow cytometry 30 min after incubation. Comparison with control of cells cultured in normal medium was by Student's *t* test: $^*P < 0.01$; †, not significantly different. Each value represents the mean ± S.E.M. of four determinations.

Pharmacological agents	OXO fluorescence intensity (arbitrary units)
Cells cultured in normal medium	
Control	25.8 ± 2.8
100 μM Verapamil	27.0 ± 2.4†
1×10^{-4}% Triton	58.8 ± 7.2*
Cells cultured in 50 mM Leu medium	
Control	23.2 ± 2.6†
100 μM Verapamil	25.9 ± 4.9†
150 μM DCCD	24.9 ± 4.9†
1×10^{-4}% Triton	48.2 ± 2.4*

Table 2 Effect of pharmacological agents on intracellular ATP

Intracellular ATP contents were measured using luciferine-luciferase (M. Komatsubara, A. Abe, R. Okumura, S. Miyauchi and N. Kamo, unpublished work). Comparison with controls of cells cultured in normal medium was by Student's *t* test: NS, not significantly different. Each value represents the mean ± S.E.M. of four determinations.

Pharmacological agents	Intracellular ATP (nmol/mg of protein)
Cells cultured in normal medium	
Control	2.74 ± 0.61
100 μM Verapamil	2.72 ± 0.43 (NS)
Cells cultured in 50 mM Leu medium	
Control	2.96 ± 0.83 (NS)
100 μM Verapamil	2.47 ± 0.15 (NS)

remained unaltered irrespective of verapamil concentration, indicating that it directly inhibited the efflux transporter without affecting the energy system (Tables 1 and 2).

The overloaded amino acids do not affect energy levels, or membrane potential and intracellular ATP concentrations

As described above, there is also an intrinsic efflux system in the wild-type cells. The low level of RH123 accumulated by the activation of the intrinsic efflux system may be due to an increase in the energy source, ATP. We thus determined the change in energy level, or membrane potential and intracellular ATP, when cultured in normal medium and that containing a large excess of leucine (Tables 1 and 2). There was little difference in the intracellular ATP level and membrane potential between the cells cultured in the presence or absence of 50 mM leucine, implying that the enhancement of RH123 efflux in cells cultured in an large excess of amino acids is not attributable to a change in energy level, or membrane potential and intracellular ATP.

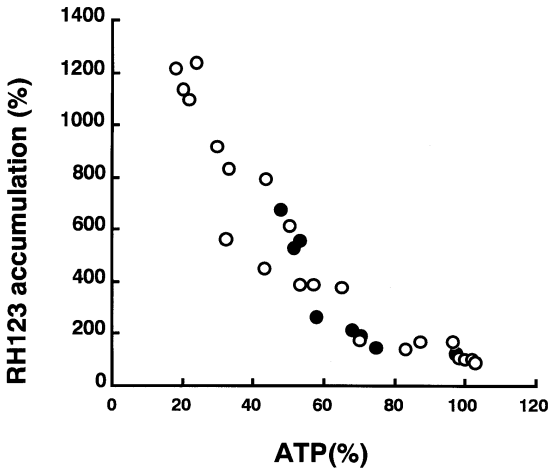

Figure 3 Relationship between RH123 accumulation and the intracellular ATP content

The experimental medium and the conditions were as described in the legend to Figure 1. ATP content and RH123 accumulation are expressed as percentages of those in cells in the absence of DCCD. Cells were incubated with various concentrations of DCCD (10–150 μM) at 4 °C overnight. Intracellular ATP contents were measured using luciferine-luciferase (M. Komatsubara, A. Abe, R. Okumura, S. Miyauchi and N. Kamo, unpublished work). Open and closed symbols represent DOX-resistant mutant cells and cells cultured in the presence of 50 mM leucine. Data on the DOX-resistant mutant cells were from (M. Komatsubara, A. Abe, R. Okumura, S. Miyauchi and N. Kamo, unpublished work).

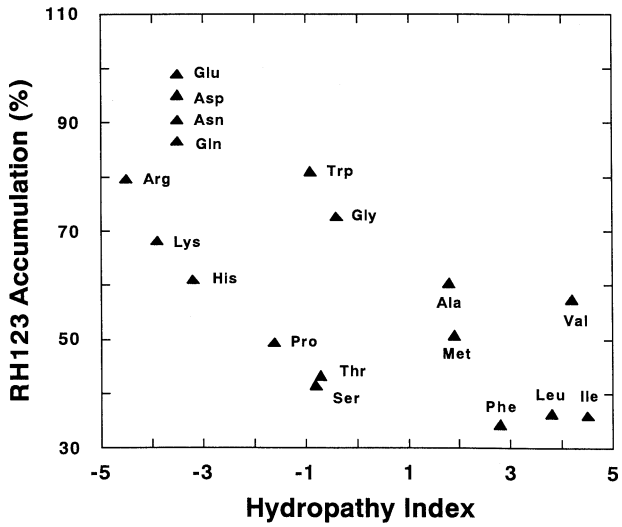

Figure 4 Steady-state RH123 accumulation of cells cultured in the presence of an excess of various amino acids versus their hydropathy index (i.e. hydrophobicity of amino acids)

Data were taken from Table 4 and the hydropathy index values were from ref. [9].

The efflux system reveals ATP-dependence

To determine the effect of different intracellular ATP concentrations on RH123 accumulation, or the efflux activity, cells cultured in the medium containing 50 mM leucine were incubated with an ATPase inhibitor, DCCD, at various concentrations. Figure 3 shows a negative correlation between RH123 accumulation and the intracellular ATP level. In contrast, OXO fluorescence intensity, which reflects membrane potential, rem-

Table 3 Effect of pharmacological agents on RH123 accumulation

Pharmacological agents (final concentration, 20 μM) were added to cell suspensions (0.1 mg of protein/ml) which had been incubated in the uptake buffer containing 0.22 μM RH123 for 15 min. RH123 accumulation was determined 15 min after adding the pharmacological agents. Comparison with control was by Student's t test: $^{*}P < 0.05$; $^{**}P < 0.01$; †, not significantly different. Each value represents the mean ± S.E.M. of three determinations.

Pharmacological agents	RH123 accumulation[a] (%)
Cells cultured in normal medium	
+ Daunomycin	84.3 ± 15.4†
+ Vinblastine	84.5 ± 7.0†
+ Vincristine	140.3 ± 18.0*
+ Colchicine	162.1 ± 18.5*
Cells cultured in 50 mM Leu medium	
+ Daunomycin	188.7 ± 19.3**
+ Vinblastine	243.1 ± 18.1**
+ Vincristine	373.5 + 39.1**
+ Colchicine	402.3 ± 24.0**
DOX-resistant mutant cells[b]	
+ Daunomycin	238.0 ± 7.1**
+ Vinblastine	274.1 ± 11.9**
+ Vincristine	547.0 ± 19.0**
+ Colchicine	417.3 ± 28.8**

[a] RH123 accumulation was expressed as the percentage of fluorescence intensity with reference to the intensity before the addition.

[b] Data on DOX-resistant mutant cells were quoted from ref. [1].

Table 4 Effect of various amino acids in the culture medium on RH123 accumulation

The concentration of the amino acids was 50 mM, except for Trp (10 mM) and Phe (25 mM). The experimental medium and conditions were the same as those described in the legend to Figure 1. Comparison with control was by Student's t test. $^{*}P < 0.05$; $^{**}P < 0.01$; †, not significantly different. Each value represents the mean ± S.E.M. of three to six determinations.

Amino acids added	RH123 accumulation (% of control)
Control	100 ± 11.3
+ Leu	36.39 ± 7.12**
+ Ile	35.98 ± 11.13**
+ Phe	34.38 ± 14.2**
+ Ser	41.65 ± 7.45**
+ Thr	43.29 ± 8.06**
+ Met	50.83 ± 5.85**
+ Val	57.83 ± 3.0**
+ Pro	49.38 ± 10.41**
+ His	61.10 ± 8.17**
+ Gly	72.77 ± 6.75*
+ Ala	60.47 ± 11.92**
+ Lys	68.26 ± 9.34*
+ Arg	79.62 ± 6.82*
+ Trp	81.08 ± 6.7†
+ Gln	86.56 ± 0.82†
+ Asn	90.55 ± 10.2†
+ Asp	95.10 ± 14.1†
+ Glu	99.01 ± 0.80†

ained unchanged irrespective of DCCD concentration, in the presence of which intracellular ATP was completely depleted (Tables 1 and 2). DCCD seems to have elicited a minimal change in the membrane potential. A H^+ electrochemical potential difference across the membrane thus appears to be uninvolved in the energization of the RH123 efflux. Na^+ and Cl^- electrochemical potential differences might also remain unchanged, since the former is driven through the Na^+/H^+ antiporter by a H^+ electrochemical potential gradient, and the latter is due mainly to the membrane potential [7,8]. Consequently, the strict relationship between the intracellular ATP concentration and RH123 accumulation under conditions maintaining an ion electrochemical potential difference indicated the involvement of ATP in the energization of the efflux system.

The efflux system resembles the DOX-induced system

The maintenance of the low intracellular RH123 concentration was reversed by the anti-cancer drugs daunomycin, vinblastine, vincristine and colchicine (Table 3) which the DOX-efflux transporter in this bacterium recognizes and expels, as described [1]. The drug spectrum of inhibition in the DOX-induced efflux system is also summarized in Table 3, revealing the good agreement among these spectra. As shown in Figure 3, the relationship between intracellular ATP and RH123 accumulation when the cells were cultured with a large excess of amino acids also resembles that of the efflux system in the DOX-resistant mutant (M. Komatsubara, A. Abe, R. Okumura, S. Miyauchi and N. Kamo, unpublished work). The efflux system, similar to that in DOX-resistant mutant, might be additionally induced when cultured with an excess of amino acids.

RH123 efflux is induced when cultured in a large excess of various amino acids or under imposed 'metabolic stress'

We examined which amino acids in the culture medium could induce the transporter. We measured the RH123 accumulated by the cells grown in the presence of various excess amino acids, and the results are summarized in Table 4. Most amino acids reduced levels of RH123 accumulation; hydrophobic amino acids, i.e. Leu, Ile and Phe, were the most effective, while Asp, Trp, Gln, Asn and Glu were the least effective. Thus hydrophobicity may be involved in the induction of RH123 efflux. RH123 accumulation was then plotted against the hydropathy indices of the amino acids [9] (Figure 4). The smaller the hydropathy index an amino acid had, the less it decreased the RH123 accumulation (minimal activation of the transporter). Owing to poor solubility, lower concentrations of Trp and Phe were added. In terms of activating the transporter, there were two groups of amino acids: one contains Arg, Lys, His, Pro, Thr and Ser, and the other, Trp, Gly, Ala, Met, Phe, Leu and Ile. Although the reason for this hypothesis remains unanswered, it appears that hydrophobicity is not the only factor affecting activation and that metabolites of the amino acids in this bacterium should be considered. The reason for this is based on our findings (S. Tanabu, E. Imai, A. Abe, R. Okumura, K. Kaidoh, S. Miyauchi, T. Nara and N. Kamo, unpublished work) that an excess of glucose or fructose in the culture medium activated the efflux transporter, although glucose, fructose and their analogues are very hydrophilic. Non-metabolized analogues did not activate the efflux transporter. There was also good agreement between the use of the overloaded glucose and the activity of the efflux transporter, indicating that glucose metabolism is involved in the activation. It should be stressed that hydrophobicity is not the only factor and that the metabolism of amino acids plays an important role.

Concluding remarks

In this study, we demonstrated that a drug efflux system similar to that in the DOX-resistant mutant was additionally induced

when cultured with a large excess of essential amino acids. It is notable that the efflux system was induced not only by cytotoxic compounds but also by essential factors, such as nutrients. This induction leads to the notion that the efflux system plays a role in actively expelling cytotoxic compounds or toxic metabolites generated in cells and thus in protecting cells. On the other hand, we report here only the enhancement of the efflux transporter, because, at present, we have no means with which to assay the expressed protein. Strictly speaking, whether the induced efflux transporter described here is identical to the DOX-efflux transporter in the mutant cells still remains speculative. One can also consider the other mechanisms of the efflux enhancement: (i) the overproduction of an intrinsic efflux transporter by the overloaded amino acids; (ii) a newly induced efflux transporter different in character from the intrinsic efflux transporter; and (iii) the synthesis of an activator of the intrinsic efflux transporter induced by the overloaded amino acids. Further investigation using a clone of the efflux transporter gene or purification of the transporter with subsequent production of an antibody is required to address the mechanism of the enhancement in more detail.

We postulate that metabolites derived from amino acids or sugars also induce the efflux transporter. A large excess of nutrients in the culture medium would produce metabolites that may stress the cells. In this sense, cells cultured in the presence of a large excess of nutrients may be referred to as metabolically stressed, which induces the efflux transporter. To prove the above hypothesis, we have to clarify how the homoeostasis is disturbed by the overloaded amino acids, or what metabolite causes changes in homoeostasis such as morphological and biological changes. However, it is very difficult to identify this mechanism, since how amino acids are metabolized in this bacterium is relatively unknown. The metabolic pathways of amino acids in this bacterium await further investigation to clarify the activation of the transporter.

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