Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm

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Stable associations of more than one species of symbiont within a single host cell or tissue are assumed to be rare in metazoans because competition for space and resources between symbionts can be detrimental to the host[1]. In animals with multiple endosymbionts, such as mussels from deep-sea hydrothermal vents[2] and reef-building corals[3], the costs of competition between the symbionts are outweighed by the ecological and physiological flexibility gained by the hosts. A further option for the coexistence of multiple symbionts within a host is if these benefit directly from one another, but such symbioses have not been previously described. Here we show that in the gutless marine oligochaete

***Olavius algarvensis*, endosymbiotic sulphate-reducing bacteria produce sulphide that can serve as an energy source for sulphide-oxidizing symbionts of the host. Thus, these symbionts do not compete for resources but rather share a mutualistic relationship with each other in an endosymbiotic sulphur cycle, in addition to their symbiotic relationship with the oligochaete host.**

Olavius algarvensis[4] is a small tubificid worm (0.2 mm × 20–30 mm) that is found in the Mediterranean at sediment depths of 5–15 cm in coarse-grained sands surrounding beds of sea grass. As in other gutless oligochaetes[5,6], two bacterial morphotypes occur in immediate proximity to one another just below the cuticle between extensions of the epidermal cells (Fig. 1). The larger morphotype (2.5 μm × 1.5 μm) contains numerous intracellular globules, whereas the smaller (1.1 μm × 0.7 μm) has no conspicuous inclusions.

We determined the phylogenetic identity of the *O. algarvensis* symbionts by using comparative 16S ribosomal RNA sequencing. We identified two dominant clone groups in the hosts, with minimal variations in the 16S rRNA sequences within each clone group (0.1–1.2%). Phylogenetic analyses revealed that the 16S rRNA sequences from these two groups are derived from the γ- and δ-subclasses of the Proteobacteria (Fig. 2a, b). The γ-proteobacterial sequence isolated from *O. algarvensis* consistently falls in a cluster with endosymbionts from other gutless oligochaetes such as *Olavius loisae*[7] and *Inanidrilus leukodermatus*[8] (96–97% sequence identity) in all treeing methods used. The δ-proteobacterial sequence is always placed within a subgroup of free-living sulphate-reducing bacteria (*Desulfococcus*/*Desulfonema*/*Desulfosarcina*) by all inference methods, with *Desulfosarcina variabilis* consistently identified as its closest relative (93% sequence identity).

Fluorescence *in situ* hybridization (FISH) confirmed that the γ- and δ-proteobacterial 16S rRNA sequences originated from the symbiotic bacteria in *O. algarvensis* (Fig. 3). The FISH signal from the probe specific to the γ-subclass of the Proteobacteria (GAM42a) and a species-specific probe based on the *O. algarvensis* γ-sequence (OalgGAM445) clearly originated from the larger bacterial symbiont, whereas the general *Desulfosarcina*/*Desulfococcus* probe (DSS658) and a probe targeting the *O. algarvensis* δ-sequence (OalgDEL136) consistently labelled the smaller bacterial symbiont.

The thioautotrophic nature (that is, sulphur-oxidizing, CO_2-fixing metabolism) of the γ-symbionts in *O. algarvensis* is suggested by their close evolutionary relationship to symbionts already characterized as thioautotrophic[8,9]. This assumption is corroborated by our results from immunocytochemical labelling with an antiserum directed against form I of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the key CO_2-fixing enzyme. The antiserum consistently labelled the larger γ-symbionts but not the smaller δ-symbionts (see Supplementary Information). Further evidence for a thioautotrophic metabolism of the γ-symbionts is

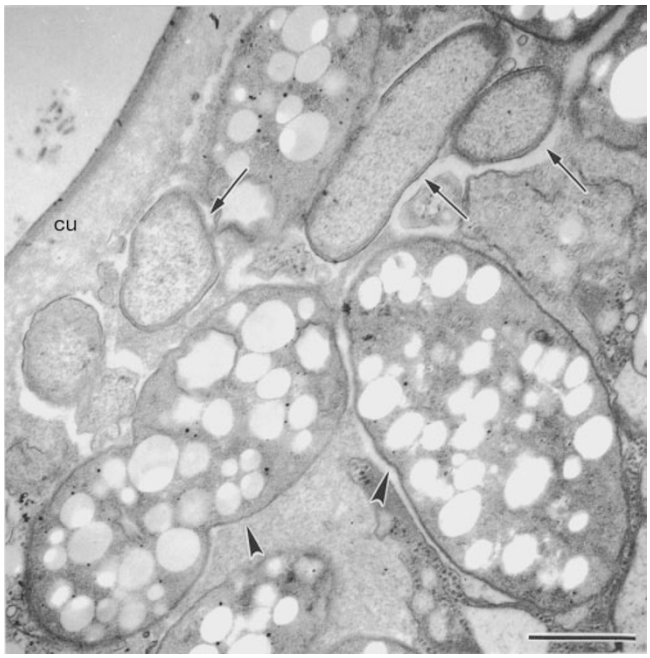

Figure 1 Transmission electron micrograph of bacterial endosymbionts in *O. algarvensis*. The symbionts occur just below the cuticle (cu) between extensions of the epidermal cells. The larger bacteria (arrowheads) contain numerous globules whereas the smaller bacteria (arrows) do not show any cytoplasmic inclusions. Scale bar, 1 μm.

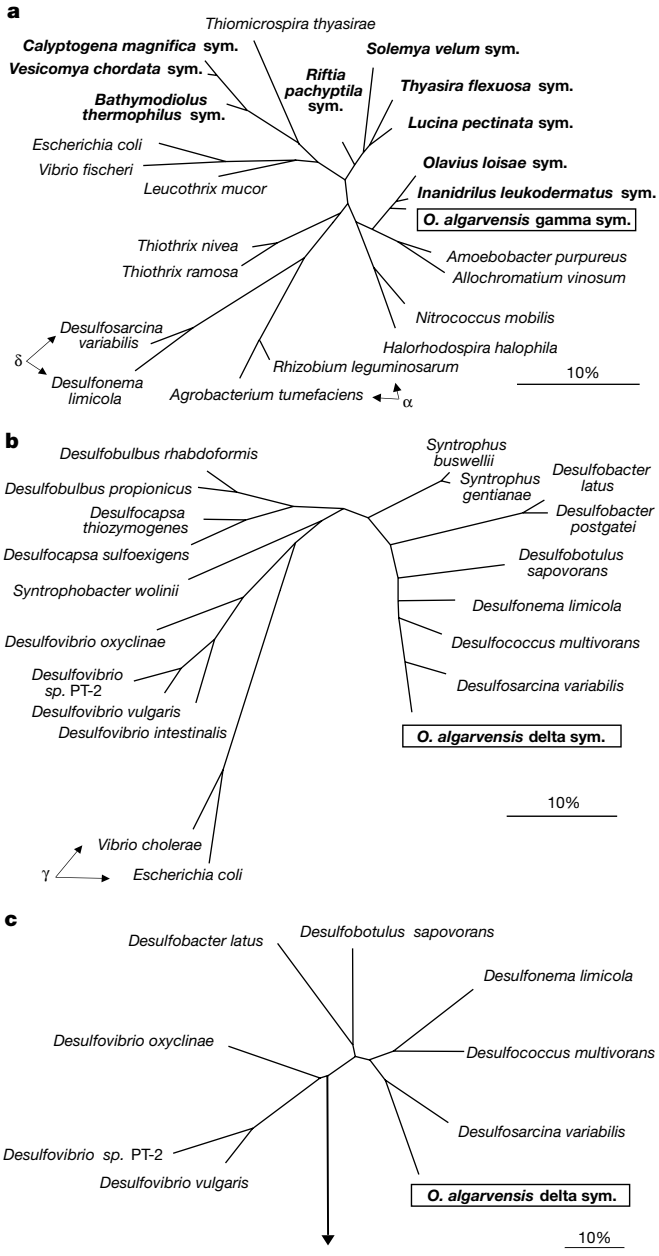

Figure 2 Phylogenetic relationships of the *O. algarvensis* symbionts based on maximum likelihood analyses. **a**, 16S rRNA sequences from the γ-subclass of Proteobacteria (α- and δ-Proteobacteria marked with arrows; chemoautotrophic symbionts (sym.) in bold type). **b**, 16S rRNA sequences from the δ-subclass of Proteobacteria (γ-Proteobacteria marked with arrows). **c**, DSR sequences based on a concatenated amino-acid alignment encompassing the DSR α- and β-subunit data sets. Arrow indicates published[16] and unpublished DSR sequences (M.W. *et al.*) not shown in tree. Scale bars indicate 0.10 expected substitutions per site.

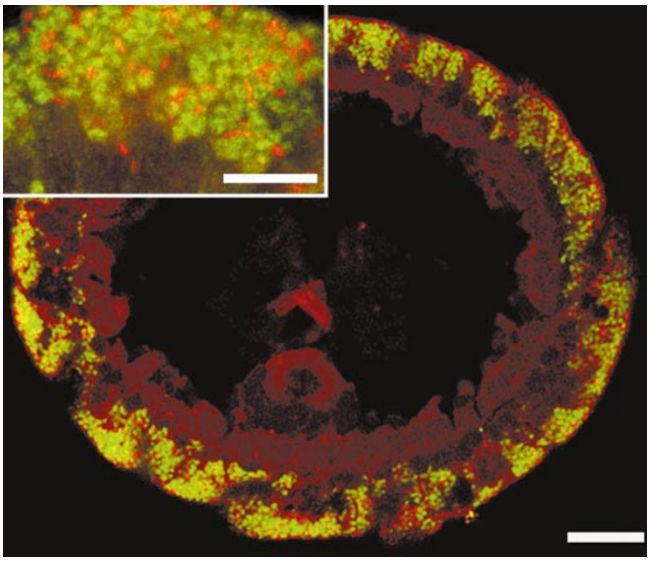

Figure 3 Fluorescence *in situ* hybridization of endosymbionts in *O. algarvensis* with oligonucleotide probes labelled with fluorochromes. γ-symbionts are green, δ-symbionts are red. Cross-section through entire worm. Hybridization with probes for the γ-subclass of the Proteobacteria and the *Desulfosarcina/Desulfococcus* group. Scale bar, 20 μm. Inset: body wall of the worms with symbiont-containing region. Hybridization with specific probes for the γ- and δ-symbionts. Scale bar, 10 μm.

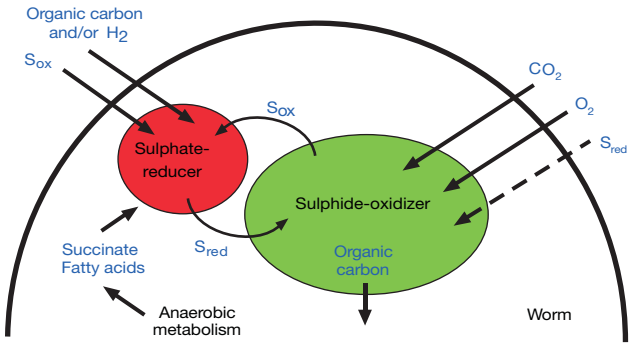

Figure 4 Model of the endosymbiotic sulphur cycle in *O. algarvensis* showing syntrophic cycling of oxidized and reduced sulphur compounds between the sulphate-reducing and sulphide-oxidizing symbionts. Under typical conditions of low sulphide flux from the sediment, sulphide produced internally by the sulphate reducers is used by the sulphide oxidizers as an electron donor for the autotrophic fixation of CO_2. Electron donors such as succinate and fatty acids can be supplied to the sulphate reducer internally by the worm during anaerobic metabolism. For net growth of the symbiotic association, external electron donors (organic carbon or H_2) are taken up from the sediment.

the high concentration of elemental sulphur in *O. algarvensis* (3.2 ± 1.7% dry weight; $n = 5$). Such large amounts of S^0 are characteristic for hosts with sulphide-oxidizing symbionts[10]. This corresponds well with electron microscopic spectroscopy studies that show the presence of sulphur in globules of the γ-symbionts (J.K., unpublished results).

The close evolutionary relationship of the δ-symbionts of *O. algarvensis* to free-living sulphate-reducing bacteria (SRB) suggests that these are also sulphate reducers. SRB have been described from termite guts[11] and the intestines of some mammals[12] and there is indirect evidence that they may occur as epibionts on some marine ciliates[13] and invertebrates[14,15]. However, SRB as endosymbionts have not been previously found in marine invertebrates and it has been suggested that such symbioses are unlikely because sulphide, their metabolic endproduct, is toxic to most aerobic organisms. We therefore used several methods to show that the δ-symbionts of *O. algarvensis* are indeed SRB and can actively respire sulphate in the worms.

The enzyme dissimilatory sulphite reductase (DSR) catalyses the reduction of (bi)sulphite to sulphide and is a good indicator for dissimilatory sulphate respiration, as it is only known to occur in sulphate-reducing prokaryotes[16]. Using specific primers, we successfully amplified the gene encoding DSR from *O. algarvensis*; no amplification products were obtained from negative controls with another gutless oligochaete host (*I. leukodermatus*) that does not harbour δ-proteobacterial symbionts. Comparative phylogenetic analyses (Fig. 2c) consistently showed that the DSR sequence from *O. algarvensis* is most closely related to *D. variabilis* (79% DNA sequence identity, 82% amino-acid identity), the free-living SRB most closely related to the δ-symbiont of *O. algarvensis* on the basis of 16S rRNA analyses (Fig. 2b). Previous studies have shown that 16S rRNA phylogenies of SRB agree well with their DSR phylogenies[16], indicating that the DSR sequence isolated from *O. algarvensis* originated from the δ-symbiont of this host and thus that this symbiont is a sulphate reducer.

To show that sulphate is actively reduced in *O. algarvensis*, we inserted silver needles through individual worms and incubated these in radiolabelled $^{35}SO_4^{2-}$ under microaerobic and aerobic conditions. After exposure of the needles to an autoradiographic film, blots from the needles inserted in live worms under microaerobic conditions showed a positive signal from ^{35}S-labelled sulphide that had precipitated on the needles, whereas under the same conditions a needle inserted in a formalin-fixed worm remained unlabelled (data not shown). This indicates that sulphate is reduced to sulphide during dissimilatory sulphate respiration by the δ-symbionts of *O. algarvensis* under microaerobic conditions. Sulphate respiration appears to be inhibited at high O_2 concentrations, on the basis of the absence of a sulphide precipitate on needles inserted in worms incubated under aerobic conditions.

We determined the sulphate reduction rates (SRRs) of the symbionts by incubating *O. algarvensis* in $^{35}SO_4^{2-}$ under microaerobic conditions (Table 1). In live worms, we measured SRRs of 53–

Table 1 Sulphate reduction rates in *O. algarvensis* specimens

Substrate	Worms	No. rep*	O_2 (μM)†	Sulphate reduction rate (pmol per worm per day)‡	Sulphate reduction rate (nmol cm^{-3} per day)§
Agar	Live	4	2–4	115 ± 76 (53/72/113/223)	1,474 ± 957 (690/935/1,440/2,830)
Agar	Live	1	200	<7.2	<80
Agar	Dead	1	2–4	<7.2	<80
Sand	Live	3	Micro-aerobic	638 ± 779 (115/266/1,534)	8,213 ± 9,917 (1,470/3,570/19,600)
Sand	Live	1	Aerobic	<7.2	<80
Sand	Dead	1	Micro-aerobic	<7.2	<80

Specimens were incubated in agar or sterilized sand.
*Number of replicate experiments; values in parentheses are rates measured in each replicate experiment.
† Measured at oligochaete surface in agar experiments (oxygen conditions in sand experiments are estimated from the agar experiments, as an identical experimental setup was used).
‡ Detection limit, 7.2 pmol per worm per day.
§ Detection limit: 80 nmol cm^{-3} per day.

1,534 pmol per worm per day, whereas SRRs in heat-killed worms under the same conditions were below detection limits. Sulphate was reduced to sulphide despite the absence of an external electron donor in the incubation medium. Endogenous electron donors that could have been used by the sulphate-reducing symbionts are fermentation products from the host such as succinate, propionate and acetate. These substrates accumulate during anaerobic metabolism under low oxygen concentrations in other marine tubificids[17] and many other aquatic invertebrates[18]. Under fully aerobic conditions, SRRs in live worms were below detection limits, indicating, as in the silver needle experiments, that high oxygen concentrations inhibit sulphate reduction. This corresponds well with observations on SRB in pure cultures, where most species are temporarily oxygen tolerant but not able to respire sulphate in the presence of high oxygen concentrations[19]. On the basis of the numbers of δ-symbionts in *O. algarvensis* as estimated by FISH, SRRs in these hosts (0.07–0.36 fmol per cell per day) are lower than those of SRB in pure cultures with saturating substrate concentrations (0.2-50 fmol per cell per day)[20] but in the same range as those estimated for free-living SRB in marine sediments (0.01–0.09 fmol per cell per day)[21]. SRRs in the worms on a volumetric basis are extremely high (690–19,600 nmol cm^{-3} per day) and comparable with rates measured in microbial mats (2,880–43,200 nmol cm^{-3} per day)[22].

To estimate the importance to the sulphide-oxidizing symbionts of internally produced sulphide compared with the import of external sulphide from the sediment, we compared the fluxes from these two sulphide sources. Dissolved sulphide concentrations in pore waters of *O. algarvensis* collection sites were extremely low: <14–76 nM (26 ± 21, $n = 9$) at 5–15 cm sediment depth, with no trend with sediment depth or location. Correspondingly, sulphide flux from the environment into the worm was <50–270 pmol per worm per day (93 ± 75, $n = 9$). Internal sulphide production from the sulphate-reducing symbionts on the basis of SRRs was 120–1,530 pmol per worm per day (640 ± 780, $n = 3$). Thus, internal sulphide production is typically considerably higher than sulphide flux from the sediment, indicating that under prevalent conditions this symbiosis appears to be independent of an external source of sulphide.

The coexistence of sulphate-reducing and sulphide-oxidizing bacteria as endosymbionts in *O. algarvensis* indicates that these are engaged in a syntrophic sulphur cycle in which oxidized and reduced sulphur compounds are recycled between the two symbionts (Fig. 4). For net growth of the symbiotic association, uptake of organic or inorganic sources of carbon and electron donors from the environment is required. As sulphide flux calculations indicate that the electron donor for the sulphide oxidizers is typically supplied internally, external reductants must be imported through the sulphate reducers. Given the metabolic diversity of SRB, in particular within the *Desulfosarcina* group, where both chemo-organotrophic and chemoautotrophic metabolism occurs, dissolved organic carbon and hydrogen are possible sources of reducing power. Migration of the worms between oxidized and reduced sediments, as described for other gutless oligochaetes[23], would provide the host and its sulphide-oxidizing symbionts with oxygen and the sulphate reducers with reductants. The benefits of this endosymbiotic sulphur cycle to its partners are clear. Cycling of oxidized and reduced sulphur compounds between the two symbionts would result in increased protein yields, as shown for continuous cultures with free-living SRB and sulphide-oxidizing bacteria[24]. Furthermore, fermentation products of the host that accumulate during anaerobic metabolism would provide the sulphate reducers with an ideal energy source, aid the hosts in the removal of these undesirable endproducts and recycle metabolites that would otherwise be lost to the symbiosis. A further advantage for the host and its thioautotrophic symbiont is that they are not limited by the external presence of reduced sulphur compounds, given the endogenous production of sulphide by the sulphate-reducing symbiont. Thus the uptake of a sulphate reducer may have enabled these hosts to colonize new habitats and extend their geographic distribution. □

Methods

For more details see Supplementary Information.

Specimens

O. algarvensis was collected in 1998–2000 from sediments at 6–8 m water depth in a bay off Capo di San Andrea (Elba, Italy) by SCUBA divers. *I. leukodermatus* specimens used as negative controls for the DSR amplifications were collected in Bermuda in 1997.

Pore water sulphide

Pore water was collected at 5, 10 and 15 cm depth at the *O. algarvensis* collection sites by SCUBA divers with immediate fixation of the samples in zinc acetate. In June 1999, October 1999 and January 2000, 1–2 ml of pore water per sample was collected and total sulphide concentrations were below the detection limit of 0.4 μM in all samples. In June 2000 the detection limit was lowered to 14 nM by collecting greater amounts of pore water (40–60 ml per sampling site) using samplers connected to evacuated serum vials containing zinc chloride. Concentrations of total sulphide were determined colorimetrically[25].

Transmission electron microscopy and immunocytochemistry

O. algarvensis individuals were fixed and prepared for electron microscopy as described[4]. For Rubisco immunocytochemistry, specimens were treated as described in ref. 9. In each worm ($n = 5$) 50–100 symbionts were examined for labelling response.

DNA analyses

Three *O. algarvensis* individuals (and two *I. leukodermatus* specimens for DSR controls) were prepared singly for polymerase chain reaction (PCR) as described in ref. 7. DNA was isolated from *D. variabilis* DSM 2060 as described[16]. Amplifications were performed with primers specific for the bacterial 16S rRNA genes (8F and 1507R) or the DSR genes of SRB (DSR1F and DSR4R)[16]. PCR products were cloned and grouped using amplified ribosomal DNA restriction analysis (ARDRA). Two or three clones per individual from dominant ARDRA groups were partially sequenced and at least one clone per individual from each ARDRA group was sequenced fully in both directions. Alignments, treeing and phylogenetic analyses (distance, parsimony and maximum likelihood) were performed with the ARB program (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/).

FISH

Five worms were fixed and prepared for FISH as described[7]. Sections were hybridized as described[7] with Cy3 and Cy5 labelled group-specific probes (GAM42a and DSS658) as well as two specific probes designed for this study (OalgGAM445: 5′-CTCGAGATCTTTCTTCCC-3′; OalgDEL136: 5′-GTTATCCCCGACTCGGGG-3′). Specificity of the probes was tested with reference strains as described[7].

$^{35}SO_4^{2-}$ incubations

For silver needle experiments worms were incubated in $Na^{35}SO_4^{2-}$ and 0.2-μm pore-size filtered seawater from the collection site. The medium was solidified with agar and the worms paralysed with lidocaine (2 mg ml^{-1}) to prevent excessive movements during insertion with silver needles (99.999% pure 50 μM Ag wire, tapered to a <1 μm tip). Incubations were run for 2–3 h under microaerobic (2–4 μM O_2) and aerobic (200 μM O_2) conditions with monitoring of oxygen concentrations with microsensors (two replicate experiments per O_2 concentration with one worm per incubation). In a control experiment at 2–4 μM O_2 with a dead worm, the specimen was fixed in 4% formalin in seawater and subsequently washed in filtered seawater. After removal, the needles were washed in 50 mM Na_2SO_4 solution and exposed to autoradiography film. Results were similar in replicate experiments.

For determination of SRRs we incubated five worms per experiment for 2–3 h in seawater with $Na^{35}SO_4^{2-}$ using agar or sand as substrates. Sand incubations were prepared and run in the same manner as the agar experiments (see above), but worms were not paralysed and moved freely in sand from the collection site that had been washed and combusted at 480 °C. Oxygen concentrations were not monitored during the sand incubations. For control experiments, specimens were heat killed in water at 70 °C for 10 min. SRRs were determined using the one-step acidic Cr-II method to separate reduced ^{35}S (ref. 26).

Elemental sulphur analyses

S^0 was extracted individually from five worms with methanol and quantified by high-performance liquid chromatography as described[27].

Flux calculations

Sulphide flux (Q) from the environment was calculated using the following equation[28]: $Q = 2\pi l D_{eff} C_p / \ln(1+2\delta/d)$, where the length of the worm (l) is 1 cm, the effective diffusion coefficient of total sulphide in sediment (D_{eff}) is 1.39×10^{-9} m^2 s^{-1}, C_p the concentration of total sulphide in the pore water, the mass boundary layer (δ) is 100 μm and the diameter

(d) of the worm is 200 μm (see Supplementary Information). All assumptions are conservative and result in an overestimation of sulphide flux from the sediment (see Supplementary Information). Internal sulphide production from the symbionts is based on SRRs measured in worms incubated in sand (Table 1), assuming that all sulphide produced is consumed by the sulphide-oxidizing symbionts. SRRs in the worms are assumed to be underestimated, given that no external electron donor was used and experimental conditions are suboptimal in comparison to the natural environment.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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